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(54) Title: HETEROLOGOUS G PROTEIN COUPLED RECEPTORS EXPRESSED IN YEAST, THEIR FUSION WITH G PROTEINS AND USE THEREOF IN BIOASSAY

(57) Abstract

The present invention is directed to expression vectors and yeast cells transformed therewith containing a first heterologous nucleotide sequence which codes for a G protein-coupled receptor, for example, the somatostatin receptor, and a second nucleotide sequence which codes for all or a portion of a G protein $\alpha\beta\gamma$ complex. Said heterologous protein is physically expressed in a host cell membrane in proper orientation for both stereoselective binding of ligands, as well as functional interaction with G proteins on the cytoplasmic side of the cell membrane. In some embodiments, a nucleotide sequence encoding a heterologous or chimeric Gα protein is expressed in conjunction with nucleotide sequences from the yeast G protein $\beta\gamma$ subunits. A second aspect of the present invention provides expression vectors and yeast cells transformed therewith encoding chimeric yeast/heterologous G protein coupled receptors. A third aspect of the present invention is directed to methods of assaying compounds using such expression constructs and yeast cell expression systems to determine the effects of ligand binding to the heterologous receptors expressed in the systems.

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Heterologous G protein coupled receptors expressed in yeast, their fusion with G proteins and use thereof in bioassay

Field of Invention

This invention relates to heterologous G protein-coupled receptor expression constructs, yeast cells expressing such receptors, vectors useful for making such cells, and methods of making and using same.

Background of the Invention

The actions of many extracellular signals, for example: neurotransmitters, hormones, odorants and light, are mediated by receptors with seven transmembrane domains (G protein-coupled receptors) and heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins). G proteins are comprised of three subunits: a guanyl-nucleotide binding α subunit; a β subunit; and a γ subunit [for review, see Conklin, B.R and Bourne,H.R. (1993 Cell 73, 631-641]. G proteins cycle between two forms, depending on whether GDP or GTP is bound to the α subunit. When GDP is bound, the G protein exists as a heterotrimer, the Gαβγ complex. When GTP is bound, the α subunit disassociates, leaving a Gβγ complex. Importantly, when a Gαβγ complex operatively associates with an activated G protein coupled receptor in a cell membrane, the rate of exchange of GTP for bound GDP is increased and, hence, the rate of disassociation of the bound Gα subunit from the Gβγ complex increases. The free Gα subunit and Gβγ complex are capable of transmitting a signal to downstream elements of a variety of signal transduction pathways. This fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena. For a review, see H.G. Dohlman, J. Thorner, M. Caron, and R. J. Lefkowitz, Ann. Rev. Biochem,

60, 653-688 (1991). G protein-mediated signaling systems are present in organisms as divergent as yeast and man. The yeast *Saccharomyces cerevisiae* is utilized as a model eukaryotic organism. Due to the ease with which one can manipulate the genetic constitution of the yeast *Saccharomyces cerevisiae*, researchers have developed a detailed understanding of many complex biological pathways. It has been demonstrated in numerous systems that the evolutionary conservation of protein structure is such that many heterologous proteins can substitute for their yeast equivalents. For example, mammalian Gα proteins can form heterotrimeric complexes with yeast Gβγ proteins [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590]. The G protein-coupled receptors represent important targets for new therapeutic drugs. Discovery of such drugs will necessarily require screening assays of high specificity and throughput. For example, therapeutic intervention in the somatostatin-growth hormone axis requires new chemical agents that act in a somatostatin receptor subtype-selective manner. The somatostatin receptor (SSTR) is a prototype of the seven transmembrane-domain class of receptors in mammalian cells. The cyclic tetradecapeptide somatostatin, first isolated from hypothalamus and shown to be a potent inhibitor of growth hormone release from the anterior pituitary, has been shown to have broad modulatory effects in CNS and peripheral tissues. In response to binding of somatostatin, SSTR activates a heterotrimeric G protein, which in turn modifies the activity of a variety of effector proteins including but not limited to adenylate cyclases, ion channels, and phospholipases. The effects of somatostatin are transduced through the action of gene products encoded in five distinct receptor subtypes that have recently been cloned [Strnad, J., Eppler, C.M., Corbett, M., and Hadcock, J.R. (1993) BBRC 191, 968-976; Yamada, Y., Post, S.R., Wang, K., Tager, H.S.,

Bell, G.I., and Seino, S. (1992) Proc. Natl. Acad. Sci. USA 89, 251-255; Meyerhof, W., Paust, H.-J., Schonrock, C., and Richter, D. (1991); Kluxen, F.-W., Bruns, C., and Lubbert, H. (1992) Proc. Natl. Acad. Sci. USA 89, 4618-4622; Li, X.-J., Forte, M., North, R.A., Rose, C.A., and Snyder, S. (1992) J. Biol. Chem. 267, 21307-21312; Bruno, J.F., Xu, Y., Song, J., and Berelowitz, M. (1992) Proc. Natl. Acad. Sci. USA 89, 11151-11154; O'Carrol, A.-M., Lolait, S.J., Konig, M., and Mahan, L. (1992) Mol. Pharmocol. 42, 939-946]. Screening assays utilizing yeast strains genetically modified to accommodate functional expression of the G protein-coupled receptors offer significant advantages in research involving ligand binding to the somatostatin receptor, as well as a host of other receptors implicated in various disease states.

Summary of the Invention

A first aspect of the present invention is directed to expression vectors and yeast cells transformed therewith, containing a first heterologous nucleotide sequence which encodes for a G protein-coupled receptor, for example, the somatostatin receptor, and a second nucleotide sequence which encodes for all or a portion of a G protein $\alpha\beta\gamma$ complex. In certain embodiments, all or a portion of a nucleotide sequence encoding for a heterologous G protein α subunit is fused to a nucleotide sequence from the yeast G protein α subunit. In certain preferred embodiments, the expression vectors and transformed cells contain a third heterologous nucleotide sequence comprising a pheromone-responsive promotor and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith. The vectors and cells may further contain several mutations. These include 1) a mutation of the *yeast SCG1/GPA1* gene, which inactivates the

yeast Gα protein, facilitating interaction of the heterologous receptor with the G protein; 2) a mutation of a yeast gene to inactivate its function and enable the yeast cell to continue growing in spite of activation of the pheromone response signal transduction pathway, preferred embodiments being mutations of the *FAR1* and/or *FUS3* genes; and, 3) a mutation of a yeast gene, the effect of the which is to greatly increase the sensitivity of the response of the cell to receptor-dependent activation of the pheromone response signal transduction pathway, preferred genes in this regard being the *SST2, STE50, SGV1, STE2, STE3, PIK1, AFRI, MSG5,* and *SIG1* genes.

A second aspect of the present invention is a chimeric expression construct and yeast cells transformed therewith comprising a first nucleotide sequence encoding for a yeast G protein coupled receptor in operative association with a heterologous nucleotide sequence which encodes for a heterologous G protein coupled receptor. The constructs and cells may contain a second heterologous nucleotide sequence comprising a pheromone-responsive promotor and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith. The constructs and cells may further contain several mutations. These include 1) a mutation of a yeast gene to inactivate its function and enable the yeast cell to continue growing in spite of activation of the pheromone response signal transduction pathway, preferred embodiments being mutations of the *FAR1* and/or *FUS3* genes; and, 2) a mutation of a yeast gene, the effect of the which is to greatly increase the sensitivity of the response of the cell to receptor-dependent activation of the pheromone response signal transduction pathway, preferred genes in this regard being the *SST2, STE50, SGV1, STE2, STE3, PIK1, AFRI, MSG5,* and *SIG1* genes. A productive signal is detected in a bioassay through coupling of the heterologous receptor to a

yeast protein.

A third aspect of the present invention is a method of assaying compounds to determine effects of ligand binding to the heterologous receptors by measuring effects on cell growth. In certain preferred embodiments, yeast cells of the kind described above are cultured in appropriate growth medium to cause expression of heterologous proteins, embedded in agar growth medium, and exposed to compounds applied to the surface of the agar plates. Effects on the growth of embedded cells are expected around compounds that activate the heterologous receptor. Increased growth may be observed with compounds that act as agonists, while decreased growth may be observed with those that act as antagonists.

Brief Description of the Drawings

FIGURE 1. Strains containing the indicated Gα expression plasmids are treated with mating pheromone (α factor). A measure of resulting signal transduction is provided by a reporter plasmid carrying *FUS1-lacZ*. Data are represented as a percent of β-galactosidase activity measured in a strain expressing solely the wild type Gα protein.

FIGURE 2. Strains containing the indicated CUP1p-Gα expression plasmid and grown in medium containing the indicated concentration of copper are treated with mating pheromone (α factor). A measure of resulting signal transduction is provided by a reporter plasmid carrying FUS1-lacZ. Data are represented as a percent of β-galactosidase activity measured in a strain expressing no exogenous Gα protein.

FIGURE 3. Saturation binding of ^{3}H-spiperone to yeast membrane fractions prepared from a strain (CY382)

expressing the 5HT1a serotonin receptor. Bmax = 3.2 pmol/mg protein; Kd - 115 nM.

FIGURE 4. Amino-terminal chimeric Ste2/5HT1a receptors. The CHI11 receptor contains the first 14 amino acids of the yeast Ste2 protein. The CHI17 receptor has a replacement of the amino-terminus of the 5HT1a receptor through the first two transmembrane domains with the corresponding region of the Ste2 receptor. The CHI18 receptor has the same Ste2 sequences fused directly to the amino-terminus of the 5HT1a receptor to create a receptor predicted to span the cellular membrane nine times. Bmax values were determined by measuring maximal binding of the radiolabeled ligand 3H-spiperone. Values are given as pmol radioligand bound per mg total protein.

FIGURE 5. Competition binding analysis of the agonists isoproterenol or epinephrine against ^{125}I-cyanopindolol with crude membrane extracts prepared from a wild-type yeast strain expressing the β_2-adrenergic receptor. Data are presented as percent maximal radioligand binding. IC_{50} values = 10 nM, isoproterenol; 200 nM, epinephrine.

FIGURE 6. Competition binding analysis of the agonists isoproterenol or epinephrine against ^{125}I-cyanopindolol with extracts prepared from a yeast strain coexpressing the β_2-adrenergic receptor and mammalian $G_{\alpha s}$. Data are presented as percent maximal radioligand binding. IC_{50} values = 10nM,isoproterenol; 60 nM, epinephrine.

FIGURE 7. Saturation binding of [^{125}I]tyr^{11}S-14 to membranes of yeast cells coexpressing the SSTR2 subtype and Scg1/$G_{i\alpha 2}$. Membranes from yeast cells expressing the SST2 subtype were prepared as described in *Experimental Procedures*. Saturation binding was performed with 20-1600 pM [^{125}I]tyr^{11}S-14. Non-specific binding for each point as cpm bound in the presence of 1 μM cold S-14 ranged from 10 to 40%. Displayed is a representative experiment performed

in duplicate.

FIGURE 8. Immunoblot showing somatostatin receptor expression. Membrane fractions are isolated from the indicated yeast strains. Aliquots of 5 to 30 µg of protein are examined by polyacrylamide gel electrophoresis/Western blot analysis. Molecular weight markers are indicated in kilodaltons. Arrows mark somatostatin receptor protein bands. Several species of this receptor are observed; doublet and triplet bands in addition to the 43 kd single receptor. Lanes, 1+2, CY602 (see Table 1); 3+4. CY603; 5+6, CY624; 7, congenic strain expressing no receptor.

FIGURE 9. Immunoblot showing muscarinic acetylcholine receptor (mAchR) expression. Membrane fractions are isolated from the indicated yeast strains. Aliquots of 30 µg of protein are examined by polyacrylamide gel electrophoresis/Western blot analysis as described in the text. Molecular weight markers are indicated in kilodaltons. Arrows mark mAchR protein bands.

FIGURE 10. Immunoblot showing α2-AR expression. Membrane fractions are isolated form the indicated yeast strains. Aliquots of 30 µg of protein are examined by polyacrylamide gel electrophoresis/ western blot analysis. Molecular weight markers are indicated in kilodaltons. Arrows mark α2-AR protein bands.

FIGURE 11. Somatostatin receptor expression plasmid, pJH1.

FIGURE 12. G protein expression plasmid, pLP82.

FIGURE 13 (A&B). Dose dependent growth response of yeast cells to somatostatin. Cultures of yeast strain LY268 are embedded in agar (top plate) or spread evenly on the surface of agar plates (bottom plate) and exposed to the indicated amounts of designated compounds spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE 14(A,B,C,&D). Growth response of yeast strains exposed to somatostatin is dependent on amount of chimeric G protein expressed. Cultures of yeast strains described in the text are embedded in agar and exposed to the indicated amounts of designated compounds spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE15(A,B,C,&D) Growth response of yeast strains exposed to somatostatin is dependent on amount of yeast G protein expressed. Cultures of yeast strains described in the text are embedded in agar and exposed to the indicated amounts of designated compounds spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE 16 (A&B). Yeast cells bearing a mutation in the *sst2* gene exhibit elevated resistance to AT when exposed to mating pheromone. Cultures of yeast strains are embedded in agar and exposed to the indicated amounts of α mating factor spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE 17 (A,B,C,&D). A mutation in the *sst2* gene enhances the growth of the yeast cells exposed to somatostatin. Cultures of yeast strains described in the text are embedded in agar and exposed to the indicated amounts of designated compounds spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE 18. Ligand-binding to the rat CCK_B receptor expressed in yeast. Crude membrane fractions from overnight liquid cultures of LY631 cells were prepared, and agonist saturation binding assays conducted as described in Methods and Materials. Saturation binding was performed with 4-60 nM[^{3}H] CCK-8 (25 µg protein/tube). Non-specific binding for each point as cpm bound in the presence of 1 µM cold CCK-8 ranged from 20 to 60%. Displayed is a representative experiment performed in duplicate.

FIGURE 19 (A&B). Growth of yeast in response to CCK_B

receptor agonists. Yeast strains that functionally express the rat CCK_B receptor (LY628, LLY631) were cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of DMSO containing 10 µl amounts of the indicated compounds. The plates were then incubated at 30°C for 3 days. **(A)** CCK-8, **(B)** CCK-4.

FIGURE 20. A_{2a}-adenosine receptor saturation binding assay. Radioligand binding assays were performed in 96-well microliter plates using binding buffers (50nM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.25% BSA) containing protease inhibitors (5µg/ml leupeptin, 5 µg/ml aprotinin, 100 µg/ml bacitracin, and 100 µg/ml benzamidine). All components were diluted in binding buffer containing protease inhibitors and added to the microliter plate wells in the following order: binding buffer, cold competitor (NECA, 1 µM final concentration), [^{3}H]NECA (1-50nM). Binding reactions were initiated by adding 82 µg of membrane protein in a 170 µl volume. Final reaction volume was 200 µl/well. All incubations were carried out at room temperature for 2 hours. Free radioligand was separated from bound ligand by rapid filtration through a glass fiber filter using an Inotech cell harvester. The filter disks were then washed several times with cold (4°C) binding buffer lacking BSA prior to counting.

FIGURE 21. Growth of yeast in response to A_{2A}-adenosine receptor agonists. LY595 cells cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of DMSO containing the 10 µg of the indicated compounds. The plates were then incubated at 30°C for 3 days. **(A, B)** CGS-21680, **(B)** NECA **(C)** DPMA.

FIGURE 22. Growth of yeast cells containing SSTR5 in response to somatostatin receptor agonists. LY620 cells cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the indicated compounds. The plates were then incubated at 30°C for 3 days. **(A)** 60 nmol S-14, (B) 30 nmol S-28.

Figure 23. Growth of yeast cells containing porcine SSTR2 in response to somatostatin receptor agonists. LY474 (two independent isolates: 21,22) were cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the indicated compounds. The plates were then incubated at 30°C for 3 days. **(1)** 600 pmol, **(2)** 60 pmol.

FIGURE 24. Deletion of *MSG5* increases the sensitivity of the yeast bioassay. Cultures of yeast strains were induced to express the SSTR2 as described in Materials and Methods and were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of S-14. The plates were then incubated at 30°C for 3 days. **(A)** MPY459 *sst2ΔADE2 msg5ΔLEU2*, **(B)** MPY458 *SST2 msg5ΔLEU2*, **(C)** LY288 *SST2 MSG5*, **(D)** LY268 *sst2ΔADE2 MSG5*.

FIGURE 25 (A&B). Growth of yeast in response to GRF receptor agonists. CY990 cells cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and

saturated with 10 µl of sterile water containing 20 nmol of the indicated compounds. The plates were then incubated at 30°C for 3 days. (**A**) hGRF(1-29)-NH_2, (**B**) hGRF (1-29), (D-arg^2)-hGRF(1-29).

FIGURE 26 (A&B). Effect of *STE50* overexpression on SSTR2 bioassay. Assay medium and yeast strains were prepared as described in Materials and Methods. Plate A contains the *STE50* overexpression strain CY560; plate B contains the control strain CY562. Filter discs saturated with solutions of the following peptides were applied to each plate: 1 mM yeast α pheromone (lefthand center), 1 µg/ml somatostatin-14 (righthand top), 100 µg/ml somatostatin-14 (righthand bottom). Plates were incubated at 30° C for 3 days.

FIGURE 27 (A&B). Bioassay of compounds with somatostatin receptor and/or antagonist properties. LY364 cells were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). For assay of antagonists, somatostatin (20 nM S-14) was added to the molten agar prior to pouring. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing test compounds. The plates were then incubated at 30°C for 3 days **(Left)** Assay for somatostatin agonists. Somatostatin (S-14) was applied to positions on the bottom row, left side (6 nmol, 600 pmol, 60 pmol, 600 pmol), **(Right)** Assay for somatostatin antagonists. Somatostatin (S-14) was applied to positions on the bottom row, left side (6 nmol, 600 pmol, 60 pmol, 600 pmol).

FIGURE 28 (A&B) Fusion of STE2 sequences to the amino terminal of SSTR2 reduces signaling effeciency in response to somatostatin. LY268 and LY322 cells were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cell/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of somatostatin (S-

14). The plates were then incubated at 30°C for 3 days. (A) LY268. S-14 was applied to filter disks clockwise from the top: carrier, 60 nmol, 6 nmol, 600 pmol, 60 pmol, 6 pmol. (B) LY322. S-14 was applied to filter disks clockwise from the top: 0.6 pmol, carrier, 60 nmol, 6 nmol, 600 pmol, 60 pmol, 6 pmol.

Detailed Description of the Invention

Nucleotide bases are abbreviated herein as follows:

A-Adenine	G-Guanine
C-Cytosine	T-Thymine
U-Uracil (sometimes herein abbreviated as "ura")	

Amino acid residues are abbreviated herein to either three letters or a single letter as follows:

Ala;A-Alanine	Leu;L-Leucine
Arg;R-Arginine	Lys;K-Lysine
Asn;N-Asparagine	Met;M-Methionine
Asp;D-Aspartic acid	Phe;F-Phenylalanine
Cys;C-Cysteine	Pro;P-Proline
Gln;Q-Glutamine	Ser;S-Serine
Glu;E-Glutamic acid	Thr;T-Threonine
Gly;G-Glycine	Trp;W-Tryptophan
His;H-Histidine	Tyr;Y-Tyrosine
Ile;I-Isoleucine	Val;V-Valine

The terms "DNA" and "nucleotide sequence" are used interchangably and are meant to include all forms of linear polymers comprising nucleotide bases, without limitation, including RNA when appropriate.

The term "mammalian" as used herein refers to any mammalian species (e.g. human, mouse, rat, and monkey).

The term "heterologous" is used herein with

respect to yeast, and hence refers to DNA sequences, proteins, and other materials originating from organisms other than yeast (e.g., mammalian, avian, amphibian, insect, plant), or combinations thereof not naturally found in yeast.

The term "upstream" and "downstream" are used herein to refer to the direction of transcription and translation, with a sequence being transcribed or translated prior to another sequence being referred to as "upstream" of the latter.

Any G protein-coupled receptor, or portions thereof, as well as the nucleotide sequences encoding same, may be employed in practicing the present invention. Examples of such receptors include, but are not limited to, adenosine receptors, somatostatin receptors, dopamine receptors, cholecystokinin receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, growth hormone releasing factor, glucagon, and serotonin receptors. The term receptor as used herein is intended to encompass subtypes of the named receptors, and mutants and homologs hereof, along with the nucleotide sequences encoding same. One skilled in the art will also understand that in some instances, it may not be necessary that the entire receptor be expressed to achieve the purposes desired. Accordingly, the term receptor is meant to include truncated and other variant forms of a given receptor, without limitation.

Any DNA sequence which codes for a Gα subunit (Gα) may be used to practice the present invention. Examples of Gα subunits include, but are not limited to Gs subunits, Gi subunits, Go subunits, Gz subunits, Gq, G11, G16 and transducing subunits. G proteins and subunits useful for practicing the present invention include subtypes, and mutants and homologs thereof, along with the DNA sequences encoding same.

One skilled in the art will understand from the teachings as presented herein that the G proteins useful in the constructs and yeast cells of the present invention may comprise neterologous Gα subunits, yeast Gα subunits, or chimeric yeast/neterologous versions. One can easily determine which configuration is best suited for adequate coupling to a particular heterologous receptor by simply constructing vectors as taught herein and measuring the signaling of ligand binding in response to a given assay. In certain preferred embodiments, $G\alpha_{i2}$ is the Gα subunit of choice, particularly when the heterologous G coupled protein is all or a portion of a somatostatin receptor. It is particularly preferred in this instance that the $G\alpha_{i2}$ subunit be coupled to a yeast Gβγ complex. Certain chimeric constructs may also provide enhanced signal transduction with regard to particular heterologous receptors. Particularly preferred is a chimeric construct formed from fusion of the amino terminal domain of yeast *GPA1/SCG1* with the carboxy terminal domain of a heterologous $G\alpha_i$, $G\alpha_s$, and especially $G\alpha_{i2}$.

Any DNA sequence which codes for a Gβγ subunit (Gβγ) may be used to practice the present invention. G proteins and subunits useful for practicing the present invention include subtypes, and mutants and homologs thereof, along with the DNA sequences encoding same. The host cells may express endogenous Gβγ, or may optionally be engineered to express heterologous Gβγ (e.g., mammalian) in the same manner as they would be engineered to express heterologous Gα.

Heterologous DNA sequences are expressed in a host by means of an expression "construct" or "vector". An expression vector is a replicable DNA construct in which a DNA sequence encoding the heterologous DNA sequence is operably linked to suitable control sequences capable of affecting the expression of a protein or protein subunit

coded for by the heterologous DNA sequence in the intended host. Generally, eukaryotic control sequences include a transcriptional promoter, however, it may be appropriate that a sequence encoding suitable mRNA ribosomal binding sites be provided, and (optionally) sequences which control the termination of transcription. Vectors useful for practicing the present invention include plasmids, viruses (including bacteriophage), and integratable DNA fragments (i.e., fragments integratable into the host genome by genetic recombination). The vector may replicate and function independently of the host genome, as in the case of a plasmid, or may integrate into the genome itself, as in the case of an integratable DNA fragment. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. For example, a promoter operable in a host cell is one which binds the RNA polymerase of that cell, and a ribosomal binding site operable in a host cell is one which binds the endogenous ribosomes of that cell.

DNA regions are operably associated when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leader sequences, contiguous and in reading phase.

Transformed host cells of the present invention are cells which have been transformed or transfected with the vectors constructed using recombinant DNA techniques and express the protein or protein subunit coded for by the heterologous DNA sequences. A variety of yeast cultures, and suitable expression vectors for transforming yeast cells, are known. See e.g., U.S. Patent No. 4,745,057; U.S. Patent No. 4,797,359; U.S. Patent No. 4,615,974; U.S. Patent

No. 4,880,734; U.S. Patent No. 4,711,844; and U.S. Patent No. 4,865,989. *Saccharomyces cerevisiae* is the most commonly used among the yeasts, although a number of other yeast species are commonly available. See. e.g., U.S. Patent No. 4,806,472 (*Kluveromyces lactis* and expression vectors therefore); 4,855,231 (*Pichia pastoris* and expression vectors therefore). Yeast vectors may contain an origin of replication from the endogenous 2 micron yeast plasmid or an autonomously replicating sequence (ARS) which confers on the plasmid the ability to replicate at high copy number in the yeast cell, centromeric (CEN) sequences which limit the ability of the plasmid to replicate at only low copy number in the yeast cell, a promoter, DNA encoding the heterologous DNA sequences, sequences for polyadenylation and transcription termination, and a selectable marker gene. Exemplary plasmids and detailed of materials and methods for making and using same are provided in the Examples section.

Any promoter capable of functioning in yeast systems may be selected for use in the constructs and cells of the present invention. Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (*PGK*) [Hitzeman et al., (1980) J.Biol. Chem. 255, 2073] or other glycolytic enzymes [(Hess et al., (1968) J. Adv. Enzyme Reg. 7, 149]; and Holland et al., (1978) Biochemistry 17, 4900], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate, decarboxylase,phosphofructokinase, glucose-6-phosphate isomerase, 3- phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publn. No. 73,657. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase, 1,2,-isocytochrome C, acid phosphates,

degradative enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization, such as the galactose inducible promoter, *GAL1*. Particularly preferred for use herein are the *PGK*, *GAL1*, and alcohol dehydrogenase (ADH) promoters. Finally, in constructing suitable expression plasmids, the termination sequences associated with these genes may also be ligated into the expression vector 3' of the heterologous coding sequences to provide polyadenylation and termination of the mRNA. In preparing the preferred expression vectors of the present invention, translational initiation sites are chosen to confer the most efficient expression of a given nucleic acid sequence in the yeast cell [see Cigan, M. and T.F. Donahue 1987, GENE, Volume 59, pp. 1-18, for a description of suitable translational initiation sites].

A particularly preferred nucleotide expression vector useful for carrying out the present invention comprises such an aforementioned promoter sequence, positioned upstream to the translational initiation site of the heterologous nucleotide sequence encoding for the heterologous G protein coupled receptor it is desired to express, and in correct reading frame therewith. Particularly preferred promoters in this regard are the *GAL1*, *PGK*, and *ADH* promoters. Positioning of the aforementioned promotor upstream to the chosen translational initiation site may enhance expression of a heterologous protein. In these preferred embodiments, no yeast G protein coupled receptor segment is fused to the heterologous G protein coupled receptor segment. The present inventors have discovered that such hybrid receptors are not critical to achieve receptor expression in yeast. This is contrary to the art accepted teaching in this regard [see King, et al. cited *infra*].

In certain other embodiments however, at least a

fragment of the 5'-untranslated region of a yeast gene is positioned upstream from the heterologous G protein coupled segment and operatively associated therewith. To that end, the present invention also provides constructs having suitable promoters and translational initiation sites as described above, but these constructs include a yeast segment comprising at least a fragment of the extreme amino-terminal coding nucleotide sequence of a yeast G protein-coupled receptor and a second segment downstream from said first segment and in correct reading frame therewith, the second segment comprising a nucleotide sequence encoding a heterologous G protein-coupled receptor. The yeast segment in this regard may be provided to actually act as a reporter sequence, rather than to serve to enhance effective expression of the heterologous G protein in the yeast system. Thus, certain embodiments comprise a gene sequence encoding a yeast segment of a yeast G protein-coupled receptor, that acts as a reporter segment, in that it encodes a peptide that may be detected through conventional means, such as antibody binding, and the like. Preferred in this regard is all or a portion of a yeast pheromone receptor fused to a heterologous G protein coupled receptor, which may be used primarily as an "epitope tag" for the highly specific detection of expression of the desired heterologous receptor using antibodies directed specifically to the epitope sequence expressed. In constructing such a vector, the yeast segment may be positioned upstream to the heterologous protein, or alternatively, a fragment of the extreme amino-terminal coding sequence of the heterologous G protein-coupled receptor may be deleted, and the yeast segment fused directly thereto. In some cases, one or more of the amino terminal transmembrane domains or intracellular domains of the heterologous protein are deleted. Alternatively, the yeast segment may be added directly to the amino terminus of the heterologous receptor, thereby

elongating the overall chimeric receptor construct.

The first and second segments are operatively associated with a promoter, such as the *GAL1* promoter, which is operative in a yeast cell. Coding sequences for yeast G protein-coupled receptors which may be used in constructing such vectors are exemplified by the gene sequences encoding yeast pheromone receptors (e.g., the *STE2* gene, which encodes the α-factor receptor, and the *STE3* gene, which encodes the **a**-factor receptor).

Certain preferred chimeric receptors provided herein comprise a yeast *Ste2* protein segment fused directly to all or a portion of a heterologous G protein receptor, and preferably, the 5HT1a receptor, muscarinic receptor, α-adrenergic receptor, or a somatostatin receptor.

Any of a variety of means for detecting the effects of ligand binding can be utilized. For example, measurement of the disassociation of Gα from Gβγ can be made through conventional biochemical techniques. However, it should be noted that the binding of ligand to a receptor may either trigger or block a detectable biological response, which may also lend itself to measurement. One such biological response is the ability of yeast cells to mate. Use of the pheromone induced mating signal transduction pathway is a preferred method of detecting the effects of ligand binding in the assay systems herein presented, the basic premise of which is discussed in more detail, as follows.

G protein-coupled pheromone receptors in yeast control a developmental program that culminates in mating (fusion) of a and α haploid cell types to form the a/α diploid (for a review, see G.F. Sprague, Jr. and J. W. Thorner, in the Molecular Biology and Cellular Biology of the Yeast Saccharomyces: volume II, Gene Expression). The process of mating is initiated by extracellular peptides, the mating pheromones. Cells of the **a** mating type secrete

α-factor, which elicits a response in α-cells; cells of the α-mating type secrete a-factor which acts only on a cells. Haploid cells respond to the presence of the peptide mating pheromones through the action of endogenous G protein-coupled pheromone receptors (*STE2*: the α-factor receptor, expressed only in α cells and *STE3*: the a-factor receptor expressed only in a-cells). Both receptors interact with the same heterotrimeric G proteins and a signal transduction cascade that is common to both haploid cell types. Upon pheromone-binding to receptor, the receptor presumably undergoes a conformational change leading to activation of the G protein. The α-subunit, *SCG1/GPA1*, exerts a negative effect on the pheromone response pathway, which is relieved by receptor-dependent activation. The complex of βγ subunits (*STE4, STE18*) is thought to transmit the positive signal to an effector, possibly *STE20*, a putative protein kinase [Leberer, E., Dignard, D., Harcus, D., Thomas, D.Y., Whiteway, M. (1992) EMBO J. 11, 4815-4824]. The effector in turn activates downstream elements of the signal transduction pathway which include *STE5*, and a presumptive protein kinase cascade composed of the products of the *STE11*, *STE7*, *FUS3* and *KSS1* genes, eventually resulting in cell cycle arrest and transcription induction. The primary interface between elements of the pheromone response pathway and cell cycle regulatory machinery is the *FAR1* gene product. Certain recessive alleles of *FAR1* and *FUS3* fail to undergo cell cycle arrest in response to pheromone, while permitting pheromone dependent transcription to occur. Pheromone-dependent transcription is mediated through the action of the sequence-specific DNA-binding protein *STE12*. Activation of *STE12* results in transcription of genes possessing a cis-acting DNA sequence, the pheromone response element. These pheromone responsive genes encode products that are required for pheromone synthesis (*MFa1, MFa2, MFA1, MFA2, STE6, STE13*) and the response to pheromone (*STE2,*

STE3, SCG1/GPA1, FUS3), facilitate or participate in cell association and fusion (*FUS1*), cell cycle arrest (*FAR1*), and the morphological events required for mating. In the event that the mating process is not consummated, yeast cells become adapted to the presence of pheromone and resume mitotic growth. Thus, in certain preferred embodiments, the *FUS3* or *FAR1* gene is mutated or deleted altogether, thereby disconnecting the cell cycle arrest pathway from the signal transduction pathway, and allowing continued growth of the cells in response to mating pheromone binding to the heterologous receptor. Since *FAR1* is a primary factor in the cell cycle regulatory pathway, its deletion or mutation is preferred in the expression constructs of the present invention. Yeast cells transformed with such constructs yield superior yeast strains for ligand-binding assays.

The mating signal transduction pathway is known to become desensitized by several mechanisms including pheromone degradation and modification of the function of the receptor, G proteins and/or downstream elements of the pheromone signal transduction by the products of the *SST2, STE50, AFR1* [Konopka, J.B. (1993) Mol. Cell. Biol. 13, 6876-6888] and *SGV1, MSG5,* and *SIG1* genes. Selected mutations in these genes can lead to hypersensitivity to pheromone and an inability to adapt to the presence of pheromone. For example, introduction of mutations that interfere with function into strains expressing heterologous G protein-coupled receptors constitutes a significant improvement on wild type strains and enables the development of extremely sensitive bioassays for compounds that interact with the receptors. Other mutations e.g. *STE50, sgv1, ste2, ste3, pik1, msg5, sig1, and afr1,* have the similar effect of increasing the sensitivity of the bioassay. One skilled in the art will understand that increased sensitivity of the assay systems is attained through deletion of one or more of these aforementioned genes, introduction of mutations that

down-regulate their expression, or in certain instances, effecting their overexpression. For example, in the *STE50* construct, overexpression of the gene is desired, not deletion of the gene.

Introduction of a constellation of mutations in the mating signal transduction pathway results in a yeast cell well suited to expression of heterologous G protein-coupled receptors, which are able to functionally respond to their cognate ligands, while providing a biological response that signals the binding of the receptor to the ligand.

In conjunction with one or more of the above-referenced mutations, a particularly convenient method for detecting ligand-binding to heterologous receptor expressed in yeast cells is to utilize a conventional genetic indicator system. Thus, in certain preferred embodiments, the cells are provided with an additional heterologous nucleotide sequence, comprising a pheromone-responsive promoter and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith. With such a sequence in place, the detecting step can be carried out by monitoring the expression of the indicator gene in the cell. Any of a variety of pheromone responsive promoters could be used, examples being promoters driving any of the aforementioned pheromone responsive genes (*e.g. mFa1, mFa2, MFA1, MFA2, STE6, STE13)*, the BAR1 gene promoter, and the FUS1 gene promoter. Likewise, any of a broad variety of indicator genes could be used, with examples including the *HIS3, G418r, URA3, LYS2, CAN1, CYH2,* and *LacZ* genes. A particularly preferred reporter gene construct is utilized by fusing transcription control elements of a *FUS1* gene to *HIS3* protein coding sequences, and replacing the original *FUS1* gene with this reporter construct. Expression of the *HIS3* gene product is thereby placed under the control of the pheromone signal transduction pathway. Yeast strains (*his3*) bearing this

construct are able to grow poorly on supplemented minimal medium lacking histidine, and are sensitive to an inhibitor of the *HIS3* gene product. In other preferred embodiments, plasmids carry a *FUS1-lacZ* gene fusion. Expression of the *FUS1* gene is stimulated in response to receptor activation by binding of pheromone. Therefore, signal transduction can be quantitated by measuring β-galactosidase activity generated from the *FUS1-lacZ* reporter gene.

Other useful reporter gene constructs, still under the control of elements of the pheromone signal transduction pathway, but alternative to the above-discussed reporter systems, may involve signals transduced through other heterologous effector proteins that are coexpressed. For example, 1) ligand-dependent stimulation of a heterologous adenylylcyclase may permit a yeast strain lacking its own adenylylcyclase due to mutation in the cdc35 gene to survive, 2) ligand-dependent stimulation of a heterologous G protein-coupled potassium channel may permit a yeast strain unable to grow in medium containing low potassium concentration [(*trk1, trk2*), for example, see Anderson, J.A. et al (1992) (Proc. Natl. Adad. Sci. USA 89, 3736-3740] to survive, or 3) ligand-dependent stimulation of a heterologous phospholipase C (especially PLC-β) may permit a yeast strain lacking its own PLC [(plc), for example, see Payne, W.E. and Fitzgerald-Hayes, M. (1993) Mol. Cell Biol. 13,4351-4363] to survive.

Any DNA sequence which codes for an adenylylcyclase may be used to practice the present invention. Examples of adenylylcyclase include the product of the D. melanogaster Rutabaga gene and the mammalian subunit types I-VIII [for review see, Tang, W.-J. and Gilman, A.G. (1992) Cell 70, 869-872], and mutants and homologs thereof, along with the DNA sequences encoding same, which are useful for practicing the present invention.

Any DNA sequence which codes for a G protein-gated

potassium channel may be used to practice the present invention. Examples of G protein-coupled potassium channel include GIRK1 [Kubo, Y. Reuveny, E., Slesinger, P.A., Jan, Y.N., and Jan, L.Y. (1992) Nature 365, 802-806], subunits useful for practicing the present invention, and mutants and homologs thereof, along with the DNA sequences encoding same.

Any DNA sequence which codes for a phospholipase protein may be used to practice the present invention. Examples of phospholipase (PLC) proteins include the D. melanogaster norpA gene product and the PLC-β proteins [for review, see Rhee, S.G., and Choi, K.D. (1992) J. Biol. Chem. 267, 12392-12396], subunits useful for practicing the present invention, and mutants and homologs thereof, along with the DNA sequences encoding same.

A particularly preferred yeast expression system is described herein, having yeast cells bearing SSTR and chimeric G-protein, and dependent upon the presence of somatostatin for continued growth. As noted above, transformed host cells of the present invention express the proteins or protein subunits coded for by the heterologous DNA sequences. When expressed, the G protein-coupled receptor is located in the host cell membrane (i.e., physically positioned therein in proper orientation for both the stereoselective binding of ligands and for functional interaction with G proteins on the cytoplasmic side of the cell membrane). Implementation of the sensitive and specific yeast expression system described herein will facilitate description of structural and functional aspects of receptor-ligand and receptor-G protein interactions. Powerful genetic selection schemes, made possible by modification of elements of the mating signal transduction pathway, may be employed to identify aspects of the receptor that have effects on agonist selectivity, ligand stereo selectivity, and determinants of agonist/antagonist binding.

The role of proteins that modify the response of receptors and G proteins to ligand may be worked out in detail with the assistance of this powerful genetic system. Importantly, the system provides a generalized approach to the study of the functioning and components of the G protein-coupled signal transduction system, as well as a generalized approach to screening assays utilizing the G protein coupled signal transduction system. The present invention provides expression constructs and assay systems adapted to receive any of a variety of heterologous G protein coupled receptors, in the form of "expression cassettes". The heterologous G protein-coupled receptor it is desired to study is simply inserted into the vectors herein provided, and expressed in yeast cells. Ligands that may bind to the expressed receptor are allowed to come into contact with the cells in any conventional assay manner, and the effects of the interaction are easily monitored. The systems presented herein thus provide tremendous utility in the identification of ligands for orphan G protein-coupled receptors and for discovering novel therapeutically useful ligands for receptors of medical, veterinary, and agricultural importance.

The following Examples are provided to further illustrate various aspects of the present invention. They are not to be construed as limiting the invention.

EXAMPLE 1

Functional expression of mammalian G α proteins in *Saccharomyces cerevisiae*.

A sensitive bioassay is utilized to measure interference of yeast Gα and Gβγ interactions by expression of heterologous Gα proteins. Mammalian Gα genes are

expressed from 2μ or centromere-bearing plasmids under the control of the constitutive *PGK* or the inducible *CUP1* promoter. The data demonstrates that the rat $G\alpha_s$, $G\alpha_{12}$, and chimeric yeast/mammalian Gα can effectively interact with yeast Gβγ.

Media and Strains. Growth of bacterial strains and plasmid manipulations are performed by standard methods (Maniatis T., Molecular Cloning, (Cold Spring Harbor Laboratory Press, 1982). Growth and transformation of yeast strains are performed as described in Rose et al. (Rose M.D., Methods in yeast genetics, Cold Spring Harbor Laboratory Press, 1990). The yeast strains used in these studies (CY414, *MATa ura3-52 trp1 leu2 his3 pep4::HIS3*) originate from strains described by E.Jones (Jones, E.W., Ann,. Rev. Genet 18:233, 1984). CY414 is sequentially transformed with the *FUS1-lacZ* fusion plasmid pSB234 (Trueheart J., et al Mol. Cell. Biol. 7(7): 2316-2328, 1987) and Gα expression plasmids.

Construction of G α expression plasmids. Rat cDNA clones for $G\alpha_s$ and $G\alpha_{12}$ and for fusions with the yeast *SCG1* gene are described elsewhere [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590]. To express these genes from low-copy-number plasmids, XhoI-SalI fragments containing each expression cassette (including the *PGK* promoter and terminator sequences) are isolated and cloned into the CEN plasmid pRS414 digested with XhoI. For inducible expression, the DNA segment containing *PGK* promoter sequences are replaced with upstream activating sequences form the *CUP1* gene.

β-galactosidase assays. Cultures are diluted to $5x10^7$ cells/ml and aliquotted into separate tubes. Pheromone is added to a final concentration of 10^{-9}M to one sample. Cultures are then incubated for 4 hrs at 30°C. Subsequent measurement of β-galactosidase activity is conducted as described elsewhere (Rose M.D., Cold Spring

Harbor Laboratory Press, 1990).

High and low-copy-number plasmids carrying the yeast *SCG1* or mammalian $G\alpha_s$ or $G\alpha_i$ or chimeric yeast/mammalian Gα genes expressed from the yeast *PGK* promoter are transformed into a wild-type yeast strain also containing a plasmid carrying a *FUS1-lacZ* gene fusion. Expression of the *FUS1* gene is stimulated in response to receptor activation by binding of pheromone. Therefore, signal transduction can be quantitated by measuring β-galactosidase activity generated from the *FUS1-lacZ* reporter gene. Interference of normal signal transduction by expression of a heterologous Gα protein is observed as a decrease in β-galactosidase activity.

Strains expressing introduced Gα genes are assayed for pheromone-induced gene activation. Data are represented as percent of wild-type response in Figure 1. Expression from all Gα plasmids reduced *FUS1-lacZ* expression levels demonstrating that the Gα proteins functionally coupled to yeast Gβγ. A dose dependence was observed for Scg1, Gαs and $G\alpha_{i2}$. Expression from high-copy-number plasmids greatly reduces signaling suggesting that a large excess of heterologous Gα protein is present. The Scg-Gαi2 chimeric protein reduces signaling to near the unstimulated background levels even from the low-copy-number plasmid. Other CEN expression plasmids reduce signaling to 53 to 84% of wild-type levels.

To achieve more precise control of Gα expression and reduce expression to a level sufficiently low that minimal effects on pheromone induced signaling will occur, Gα genes (except Gαs) are placed under the control of the inducible *CUP1* promoter and transformed into yeast on low-copy-number plasmids. The level of signaling repression mediated by these plasmids is dependent on the concentration of Cu++4 added to the medium (Figure 2). However, basal expression (no Cu++4 added) was equivalent to levels

observed from the *PGK* promoter (Figure 1). As in the previous experiment, the *SCG*-Gαi2 chimeric protein reduces signaling to almost background levels.

The data presented in Figures 1 and 2 indicates that all Gα expression plasmids examined produce functional Gα proteins in that all inhibit the signal transduction pathway. Using a constitutive promoter (*PGK*), most Gα genes exhibit a dose-dependent effect with high-copy-number 2 micron plasmids drastically reducing signaling (Figure 1). Lower expression from CEN plasmids reduce signaling levels as little as 16% (See G_i, Figure 1). Expression of the Gα genes from the CUP1 promoter shows expected dose/response effects with reduced signaling correlated to increased Cu++ concentrations (Figure 2).

EXAMPLE 2

Pharmacological evaluation of heterologous G protein-coupled receptors expressed in *Saccharomyces cerevisiae*

Yeast strains. Growth and transformation of yeast strains are performed as described (Rore, M.D., Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, 1990). The yeast strains used in these studies (CY414; *MATa ura3-52 trp1 leu2 his3 pep4ΔHIS3*) originate from strains described by E. Jones (Jones, E. W., Ann. Rev. Genet, 18:233, 1984).

Nucleic acid manipulation. Growth of bacterial strains and plasmid manipulations are performed by standard methods [Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning, 2nd ed. (Cold Spring Harbor Laboratory Press, 1989)]. DNA sequencing is performed by high temperature cycle sequencing (Applied Biosystems).

Protein analysis. Receptor expression strains are

grown in synthetic complete medium lacking specific nutrients to select for plasmid retention and containing 3% galactose to induce receptor gene expression. Cells are pelleted and washed in lysis buffer (10 mM sodium bicarbonate, pH 7.2, 1 mM EGTA, 1 mM EDTA) then resuspended in lysis buffer plus protease inhibitors (5 µg/ml leupeptin, 10 µg/ml benzamidine, 10 µg/ml Bacitracin, 5 µg/ml pepstatin, 5 µg/ml aprotinin) and lysed by physical disruption with glass beads. Debris is removed by centrifugation at 1000 X g for 10 min. The membrane fraction is isolated by centrifugation at 100,000 X g for 10 min. This pellet is washed once in lysis buffer plus inhibitors. Polyacrylamide gel electrophoresis of yeast extracts is performed by standard methods except without boiling of samples. Proteins are transferred to Immobilon-P millipore filters by the semi-dry technique. Receptor protein is visualized using ECL reagents with rabbit anti-Ste2 antibodies.

Radioligand binding assays. Reactions are performed in a volume of 0.2 µl with 5 to 50 µg of protein. Binding assays for 5HT1a receptor or β2-adrenergic receptor ligands use buffer of 50 mM Tris, pH 7.4, 10 mM $MgCl_2$. Somatostatin binding is performed in a buffer of 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$. After allowing ligand binding to reach equilibrium at room temperature, membrane fractions are isolated on GFC glass fiber filters. The following final concentrations of ligands are used: radioligands-^{3}H spiperone, 80 nM; ^{125}I-cyanapindolol, 250 pM; [^{125}I-tyr^{11}]-somatostatin 14, 250 pM; competitors-serotonin, 10 µM; propranolol, 20 µM somatostatin 14, 1 µM. The guanosine triphosphate analog Gpp(NH)p is used at 100 µM.

Expression of the human 5HT1a serotonergic receptor. The gene encoding the human 5HT1a receptor is modified to add the first 14 amino acids of the yeast Ste2 protein, cloned into the expression plasmid pMP3 and,

designated pCHI11. This strain, designated CY382, is grown in medium containing galactose to induce receptor expression, fractioned and tested for receptor activity by binding of the radiolabelled antagonist ^{3}H-spiperone. Saturation binding demonstrates that the receptor is expressed at high levels (Bmax = 3.2 pmol/mg protein) and that it binds spiperone with an affinity (Kd = 115 nM; Figure 3) similar to that observed in mammalian tissues (Kd - 20 to 100 nM).

Two chimeric receptor genes are engineered; in pCH117, sequences encoding the N-terminus including the first two transmembrane domains of the 5HT1a receptor are replaced with the corresponding sequences of the Ste2 receptor, and in pCH118, these Ste2 sequences are added directly to the N-terminus of the 5HT1a receptor to create a novel nine-transmembrane-domain receptor (Figure 4). Strains expressing these receptors are examined for binding of radiolabelled ligand. Both receptors demonstrate specific binding of the 5HT receptor antagonist ^{3}H-spiperone (Figure 4). Replacement of the first two transmembrane domains with those of an unrelated receptor does not apparently affect binding of this ligand. Addition of transmembrane domains do not effect binding, suggesting that this unusual receptor can attain a functional conformation in the cell membrane. Strains carrying pCHI11, pCHI17, or pCHI18 produce Bmax values of 3.1, 1.6, or 0.7 pmol/mg, respectively. Although these chimeric receptors produce interesting results regarding receptor structure, they do not enhance overall levels of functional receptors in the cell.

All intracellular sequences of the 5HT1a receptor are replaced with corresponding sequences of the yeast Ste2 protein to directly couple the receptor to the yeast G protein. The resultant chimeric receptor, CHI16, is expressed in a wild-type yeast strain and examined for high

affinity binding of 5HT1a receptor agonists. Agonist binding is not detected. However, the level of radiolabelled spiperone binding is equal to CHI11, indicating that this receptor is expressed at high levels and in a functional conformation.

Expression of the human β2-adrenergic receptor. The human adrenergic receptor is expressed in yeast with the intention of using it as a model to optimize expression and G protein coupling. A yeast strain expressing the receptor is examined by Scatchard analysis for binding of the ligand ^{125}I-cyanopindolol. Binding is saturable and demonstrated a Kd (23 pM), similar to that reported in mammalian tissues. Strains with or without coexpressed Gαs are then examined in competition assays in which binding of this radioligand is competed with the agonists isoproterenol or epinephrine. High affinity binding, which is only expected to occur if the receptor is actively coupled to G protein, was observed in both strains (Figures 5 and 6). The extrapolated Ki values for these ligands (isoproterenol = 10 nM; epinephrine =60 nM) are consistent with affinities observed in mammalian tissues and exhibit the expected order of potency. However, other data suggest that the high affinity binding of agonists to the β2-adrenergic receptor in yeast is anomalous and not a result of coupling to G protein. In particular, the third intracellular loop (containing the primary Gα contact points) of the β2-adrenergic receptor is replaced with the corresponding domain of the yeast Ste2 receptor. This receptor exhibits the same affinities for adrenergic agonists suggesting that the β2-adrenergic receptor takes on an inappropriate conformation in yeast.

Expression of rat somatostatin receptor. High affinity binding of somatostatin to SSTR2 is dependent on formation of a receptor/G protein complex (Strnad et al, 1993). When SSTR2 and G protein are uncoupled from each other, high affinity binding of [^{125}I]tyr^{11}S-14 is attenuated.

As shown in Figure 8, binding of [^{125}I]tyr^{11}S-14 to SSTR2 expressed in yeast coexpressing Scg1/$G_{\alpha i2}$ is saturable and of high affinity. The calculated K_d of [^{125}I]tyr^{11}S-14 binding SSTR2 expressed in yeast is 600 pM. Similar binding affinities are observed when $G_{\alpha i2}$ is coexpressed with SSTR2 rather than Scg1/$G_{\alpha i2}$. The K_d observed in yeast is in close agreement to the calculated K_d of [^{125}I]tyr^{11}S-14 binding of SSTR2 expressed in mammalian cells (Strnad et al., 1993). It is demonstrated that addition of the yeast Ste2 receptor to the N-terminus of this receptor has no affect on its ability to bind S-14. The Ste2 sequences act as a tag for immunochemical examination of receptor expression. The immunoblot shown in Figure 7 illustrates the high level of expression of SSTR2 in three different strains. Yeast strains expressing the SSTR2 somatostatin receptor and different Gα proteins are derived from strain YPH500. These strains share the genotype MATa *scg1Δ hisG lys2-801 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 ade2* SSTR2. Strains designated CY624 (Gαi2), CY602 (Gαs, and CY603 *Scg1*) are examined for specific binding of radiolabelled somatostatin-14 (S-14). All three exhibit some degree of somatostatin binding (Table 1). High affinity binding of this ligand requires coupling of the receptor to G protein, normally Gαi (Luthin D.R. 1993; Strnad J. 1993), so the high level of binding in the absence of Gαi is unexpected. As confirmation that the receptor is coupled to G proteins, binding is examined in the presence of the nonhydrolyzable guanosine triphosphate analog Gpp (NH)p. The addition of this compound eliminates ligand binding (Table 1), demonstrating that the SSTR2 receptor is coupled to G protein. These data demonstrate that a mammalian G protein-coupled receptor can functionally interact with a G protein composed of its favored Gα protein plus yeast Gβ and Gγ subunits and that a heterologous receptor can functionally couple to a G protein entirely composed of yeast subunits.

TABLE 1. Binding of ^{125}I-somatostatin

STRAIN[a]	Gα	Bmax[b]	+Gpp (NH) p[c]
CY624	Gαi	94.5	22%
CY602	Gαs	13.7	0%
CY603	Scgl	24.7	4%

[a]Crude membrane extracts were prepared from yeast strains expressing the rat SSTR2 somatostatin receptor subtype and the indicated Gα protein. The maximal binding of radiolabeled somatostatin 14 was measured as described in the text. [b]Bmax values are given as fmol/mg total protein. [c]The non-hydrolyzable GTP analog Gpp(NH)p was added to samples to uncouple receptor and G protein. Data are presented as percent radioligand bound compared to untreated samples.

Expression of *Drosophila* muscarinic acetylcholine receptor. DNA sequences encoding a *Drosophila* muscarinic acetylcholine receptor (Dm mAChR) are modified by addition of a SalI site in the 5' coding sequences through the use of PCR. DNA sequences encoding the first 23 amino acids of the *STE2* gene product are added to the 5' end of Dm mAChR as a BamHI/SalI fragment. The modified Dm mAChR is inserted into the BamHI site in plasmid pMP3, placing expression of the receptor under the control of the *GAL1* promoter, forming plasmid pMP3-Dm mAChR. Strain CY414 is transformed with this plasmid and cultured for receptor expression by standard methods. Crude membrane preparations are prepared from these cells and tested for the presence of specific binding sites for the muscarinic antagonist 3H-quinuclidinyl benzilate (10 nM) competed with atropine (50 μM). Specific binding sites (Bmax 10 and 30 fmol/mg) are observed.

Drosophila mAchR expression is also detectable by immunoblotting methods. An abundant 75 kDa polypeptide, consistent with the predicted molecular weight from the

primary sequence of mAchR, is detected in samples of protein (30µg/lane) from crude membrane preparations from cells expressing mAchR from pMP3 using an antibody directed against the associated Ste2 epitope (Figure 9). Substantially less protein is detected when mAchR is expressed from pMP2, a derivative of pMP3 lacking *GAL4* sequences, which is not expected to confirm high level expression of mAchR.

Expression of an α2-adrenergic receptor (α2-AR). An EcoRI-NarI fragment from plasmid pMP3, including the *GAL1,10* promoter EcoRI-BamHI fragment, DNA sequences encoding the first 23 amino acids of the STE2 gene product present on a BamHI-SalI fragment, SalI-SphI polylinker fragment from YEp352, and STE7 terminator sequences, is transferred to pRS424, forming pLP15. A PstI-PvuII fragment encoding a porcine α2A-AR [Guyer, C.A., Horstman, D.A., Wilson, A.L., Clark, J.D., Cragoe, E.J., and Limbird, L.E. (1990) J. Biol. Chem. 265, 17307-17317] is inserted into the PstI-SmaI sites of pLP15, forming pLP50. An EcoRI fragment of GAL4 is inserted into the EcoRI site of pLP50, forming pLP60. Strain LY124 [a derivative of YPH500 (Stratagene) containing the *scg1ΔhisG* allele and bearing plasmid pLP10 [pLP10: pUN75 (Elledge, S.J. and Davis, R.W. Genetics 87, 189-194) containing the PGK-Scg1-Gαi2 XhoI-SalI fragment from pPGKH-Scg1-Gαi2 inserted into the SalI site] is transformed with pLP60 and cultured for receptor expression by standard methods. Crude membrane preparations are prepared from these cells and tested for the presence of specific binding sites for the α2-AR antagonist 3H-rauwolscine (200 nM) competed with phentolamine (10µM). Specific binding sites with a Bmax of between 10 and 84 fmol/mg were observed.

Porcine α2AR expression is also detectable by immunoblotting methods (Figure 10). Several abundant polypeptides are detected in samples of protein (30µg/lane)

from crude membrane preparations from cells expressing α 2AR from pMP3 using an antibody directed against the associated Ste2 epitope.

EXAMPLE 3

Agonist dependent growth of yeast in response to somatotstatin receptor agonists.

Yeast strains that respond to somatostatin are created by introducing several modifications into typical laboratory yeast strains. First, a cDNA encoding the somatostatin receptor subtype 2 (SSTR2) is placed under the control of the galactose-inducible GAL1 promoter in a multicopy yeast plasmid (Figure 11). High level expression of receptor is accomplished by inducible co-overexpression of the transcriptional activating protein GAL4 from the same plasmid. Second, the endogenous Gα protein gene, GPA1/SCG1, is replaced with a chimeric gene composed of an amino terminal domain from GPA1/SCG1, and C-terminal sequences from rat Gαi2 (Figure 12). Expression of chimeric G proteins in yeast have previously been shown to suppress the growth defect of scg1/gpa1 mutant cells [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590]. Third, the FAR1 gene is deleted, permitting continued growth in the presence of an activated mating signal transduction pathway. The FAR1 protein is thought to serve as the primary interface between the mating signal transduction pathway and cell cycle machinery [Chang, F. and Herskowitz, I. (1990) Cell 63, 999-1012; Peter, M., Gartner, A., Horecka, J., Ammerer, G., and Herskowitz, I. (1993) Cell. Biol. 13, 5659-5669]. Fourth, the FUS1 gene is replaced with a reporter gene construct made by fusing transcription control elements of the FUS1 gene to HIS3

protein coding sequences, thereby placing expression of the HIS3 gene product under the control of the pheromone signal transduction pathway. Yeast strains (his3) bearing this construct are able to grow poorly on supplemented minimal medium lacking histidine and are sensitive to 3-amino-1,2,4-triazole (AT), an inhibitor of the HIS3 gene product. Receptor activation by agonist-binding leads to increased HIS3 protein expression, a corresponding increase in resistance to AT, and, therefore, the ability to grow on medium lacking histidine and/or in the presence of the inhibitor. Adjusting the pH of the growth medium to > pH 5.5 enhances the ability of such cells to grow in presence of agonist, presumably due to an increased ability of somatostatin to bind to SSTR2.

The utility of the yeast expression system lies in its adaptability to rapid mass screening. To facilitate screening for novel therapeutics directed at the SSTRs, a convenient agar plate bioassay is developed in which functional coupling of somatostatin binding to receptor and subsequent activation of the mating signal transduction pathway is detected as a zone of growth (halo) around applied compounds (Figure 13). Overnight liquid cultures of LY268 [a derivative of YPH500 (Stratagene) MATa ura3-52 lys2-801 ade2 trp1Δ63 his3Δ200 leu2Δ1 far1ΔLYS2 scg1ΔhisG fus1ΔFUS1-HIS3 sst2ΔADE2 bearing the SSTR2 expression plasmid and the SCG1-Gαi2 expression plasmid pLP82 in SC dextrose (2%) lacking ura, trp were transferred to SC Lactate medium (2%) lacking ura and trp and subsequently SC galactose (2%) medium lacking ura and trp. Cells (2 $x10^5$) are then plated in 30 ml of SC galactose (2%) lacking ura, trp, and his agar (Figure 13, top panel), or spread evenly on the surface (Figure 13, bottom panel), the indicated amounts of selected compounds applied to paper disks situated on the surface of the agar plate, and incubated at 30°C for 3-5 days. Halos of growth are observed around

disks saturated with varying concentrations of somatostatin (S-14) and MK678 [a hexapeptide analog of somatostatin that exhibits high affinity binding to SSTR2 [Verber, D., Saperstein, R., Nutt, R., Friedinger, R., Brady, P., Curley, P., Perlov, D., Paleveda, W., Zacchei, A., Cordes, E., Anderson, P., and Hirschmann, R. (1981) Life Sci, 35, 1371-1378]. Halo size increases in proportion to the amount of agonist applied and a significant response is observed even at the lowest amount applied (6 nmol S-14), demonstrating the exquisite sensitivity of the assay. No detectable response is observed with carrier alone, or to met-enkephalin, an opiate receptor agonist, demonstrating the high specificity of the assay.

Yeast medium and culture conditions are formulated according to standard procedures and DNA-mediated transformation of yeast is by the LiAc method [Sherman, F., Fink, G.R., and Hicks, J.B. (1986) Methods in Yeast Genetics (Cold Spring Harbor Laboratory Press]. LY268 is constructed by sequential insertional deletion using recombinant scg1:ΔhisG, far1ΔLYS2, FUS1ΔHIS3, and sst2ΔADE2 alleles. The scg1ΔhisG allele is assembled by inserting the hisG-URA3-hisG fragment from pNKY51 [Alani, E., Cao, L., and Kleckner, N. (1987) Genetics 116, 541-545] between the 5' EcoRI-HindIII and 3' SphI-SnaBI fragments of SCG1/GPA1. After DNA-mediated transformation of appropriate yeast strains and selections for replacement of the chromosomal allele, the URA3 gene is removed by inducing recombination between hisG repeats by growth on 5-fluoroorotic acid (FOA)-containing medium [Boeke, J., Lacroute, F., and Fink, G. (1984) Mol. Gen. Genet. 197, 345-346]. The far1ΔLYS2 allele is constructed by amplifying two fragments of the FAR1 gene [Chang, F. and Herskowitz, I. (1990) Cell 63, 999-1012] from yeast genomic DNA (strain YPH501, Stratagene) using synthetic oligonucleotides that introduce an EcoRI site at 1201 in the 5' fragment and an HindIII site at position 2017

and a SalI site at 2821 in the 3' fragment. The fragments are cloned into the EcoRI/SalI fragment of pBSK (Stratagene). The completed far1ΔLYS2 construct is digested with EcoRI and used to transform yeast. An EcoRI fragment encoding the FUS1-HIS3 reporter gene is released from pSL1497 [Stevenson, B.J., Rhodes, N., Errede, B., and Sprague, G.F. (1992) Genes Dev. 6, 1293] and used to transform appropriate yeast strains. The sst2ΔADE2 allele [Dietzel, C. and Kurjan, J. (1987) Mol. Cell. Biol. 7, 4169-4177] is built from a 2.5 kb fragment of the ADE2 gene amplified by PCR using oligos that placed a Cla site at position 1 and an NheI site at position 2518. This fragment is used to replace the internal ClaI-NheI fragment in SST2. The sst2ΔADE2 fragment is released by digestion with SalI and used to transform appropriate yeast strains.

The multistep construction of the SSTR2 expression plasmid, pJH2 (Figure 11), is initiated by inserting a SphI/NarI fragment of 3' untranslated region from the STE7 gene [Teague, M.A., Chaleff, D.T., and Errede, B. (1986) Proc. Natl. Acad. Sci. USA 83, 7371-7375], and an EcoRI/BamHI fragment of the GAL1/10 promoter [Yocum, R.R., Hanley, S., West, R., and Ptashne, M. (1984) Mol. Cell. Biol. 4 1985-1998] into appropriate sites in YEp352 [Hill, J.E., Myers 2, A.M., Koerner, T.J., and Tzagoloff, (1986) Yeast 2, 163-167.], creating pEK1. A PCR product, encoding the open reading frame and transcriptional termination sequences of the GAL4 gene, is amplified with oligos containing 5' EcoRI and 3' AatII sites and inserted into pEK1, creating pMP3. The cDNA encoding rat SSTR2 (Strnad, J., Eppler, C.M., Corbett, M., and Hadcock, J.R. (1993) BBRC 191, 968-976] is modified by PCR using oligonucleotides that add a BglII site in the DNA sequences encoding the amino terminus of SSTR2 and a BglII site directly after the translational stop site. SSTR2 coding sequences are inserted as a BglII PCR fragment into the BamHI site of pMP3.

Plasmid pLP82 (Figure 12) is constructed by first replacing the XhoI/EcoRI promoter fragment in pPGKH-SCG1-Gαs [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590], with a modified *SCG1* promoter fragment [Dietzel, C. and Kurjan, J. (1987) Cell 50, 1001-1010] amplified from yeast genomic DNA using oligonucleotides that introduce 5' XhoI and 3' EcoRI sites at positions -200 and -42, forming plasmid pLP61. The BamHI fragment encoding Gαs domain is replaced with a comparable fragment encoding Gαi2, forming plasmid pLP71. The XhoI/SalI fragment of pLP71 encoding an *SCG1*-Gαi2 chimeric G protein expressed under the control of the *SCG1* promoter is transferred to the SalI site in pRS414 (Stratagene), forming pLP82.

Plasmid pLP83 is constructed by replacing the EcoRI fragment in pLP71 with the EcoRI fragment encoding I from pPGKH-SCG1 [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590], forming plasmid pLP75. The XhoI/SalI fragment encoding SCG1 is transferred to the SalI site in pRS414 (Stratagene), forming plasmid pLP83.

EXAMPLE 4

Effects of G protein expression on the sensitivity of the bioassay.

Yeast strains LY268 (pLP82: CEN pSCG1-Scg1-Gαi2), LY262 (pRS414-PGK-Scg1-Gαi2: pRS414 containing the PGK-Scg1-Gαi2 Xho/SalI fragment from pPGKH-Scg1-Gαi2 in the SalI site), LY324 (pLP84: 2μ pSCG1-Scg-Gαi2), and LY284 (pRS424-PGK-Scg-Gαi2: pRS424 containing the PGK-Scg1-Gαi2 Xho/SalI fragment from pPGKH-Scg1-Gαi2 in the SalI site) were constructed by placing the designated plasmids in strain LY260 [a derivative of YPH500 (Stratagene) MATa ura 3-52

lys2-801 ade2 trp1D63 his3Δ200 leu2α1 far1ΔLYS2 scg1ΔhisG fus1ΔFUS1-HIS3 sst2ΔADE2 bearing the SSTR2 expression plasmid]. Overnight liquid cultures in SC-Dextrose (2%) lacking ura and trp were transferred to Sc-Lactate (2%) medium lacking ura and trp and subsequently SC-Galactose (2%) medium lacking ura and trp. Cells ($2x10^5$) are then plated in 30 ml of SC-Galactose (2%) lacking ura, trp, and his agar, the indicated amounts of selected compounds applied to paper disks situated on the surface of the agar plate, and incubated at 30°C for 3-5 days (Figure 14). The extent of growth around S-14 is dependent upon the G protein expression plasmid contained in each strain. The most luxuriant growth is observed in response to S-14 by LY268 (pLP82: CEN pSCG1-Scg1-Gαi2), less growth is seen in strains LY262 (pRS414-PGK-Scg1-Gαi2) and LY324 (pLP84: 2μ pSCG1-Scg-Gαi2), while little detectable growth is exhibited by LY284 (pRS424-PGK-Scg1-Gαi2). These results are consistent with the observed inhibition of pheromone stimulated transcriptional induction by elevated amounts of expressed Gα protein. Thus, precise regulation of G protein expression levels in strains expressing heterologous G protein-coupled receptors is critical to the design and successful implementation of a bioassay for compounds that interact with the receptor.

Plasmid pLP84 is constructed by first replacing the XhoI/EcoRI promoter fragment in pPGKH-SCG1-$G\alpha_s$ [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590], with a modified *SCG1* promoter fragment [Dietzel, C. and Kurjan, J. (1987) Cell 50, 1001-1010] amplified from yeast genomic DNA using oligonucleotides that introduce 5' XhoI and 3' EcoRI sites at positions -200 and -42, forming plasmid pLP61. The BamHI fragment encoding $G\alpha_s$ domain is replaced with a comparable fragment encoging $G\alpha_{12}$, forming plasmid pLP71. The XhoI/SalI fragment of pLP71 encoding a *SCG1*-$G\alpha_{12}$ chimeric G protein

expressed under the control of the SCG1 promoter is transferred to the SalI site in pRS424 (Stratagene), forming pLP84.

EXAMPLE 5

Somatostatin receptor is capable of transmitting signal through the endogenous yeast G α

SSTR2 is thought to couple to $G\alpha_{i2}$ and $G\alpha_{i3}$ in mammalian cells [Luthin, D. R., Eppler, C. M., and Linden, J. (1993) J. Biol. Chem. 268, 5990-5996]. However, when expressed in appropriate yeast strains (described below), SSTR2 is shown to be capable of transmitting a signal through the endogenous yeast Gα protein. Implicit in this observation is the necessary coupling of SSTR2 to the endogenous Gα protein. The ability of heterologous G protein-coupled receptors to couple to the endogenous Gα protein is a significant improvement in existing technology, and is thought not to be possible in the prior art (King K. Dohlman, H.G., Thorner, J., Caron, M.G., and Lefkowitz, R.J. (1990) Science 250, 121-123). Yeast strains LY266 (pLP83: CEN pSCG1-Scg1), LY280 (pRS414-PGK-Scg1: pRS414 containing the PGK-Scg1 Xho-SalI fragment from pPGKH-ScgI in the SalI site), LY326 (pLP86: 2μ p SCG1-Scg), and LY282 (pRS424-pPGK-Scg pRS424 containing the PGK-Scg1 Xho-SalI fragment from pPGKH-Scg1 in the SalI site) were constructed by placing the designated plasmids in strain LY260. Overnight liquid cultures of these strains, which are capable of expressing only *SCG1/GPA1*, in SC-Dextrose (2%) lacking ura and trp were transferred to SC-Lactate (2%) lacking ura, trp, and subsequently to SC-Galactose (2%) lacking ura and trp medium. Cells (2×10^5) are then plated in 30 ml SC-Galactose (2%) lacking ura, trp, and his medium, the

indicated amounts of selected compounds applied to paper disks situated on the surface of the agar plate, and incubated at 30°C for 3-5 days. Halos of growth are observed around disks saturated with varying concentrations of S-14, demonstrating that a productive signal can be transduced through an interaction between SSTR2 and the yeast *SCG1/GPA1* protein (Figure 15). These observations demonstrate that a simple and broadly applicable bioassay may be established in which any member of the class of G protein-coupled receptors may be expressed in appropriately modified yeast strains and made to couple to the endogenous Gα protein.

Plasmid pLP86 is constructed by replacing the EcoRI fragment in pLP71 with the EcoRI fragment encoding SCG1 from pPGKH-SCG1 [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10. 2582-2590], forming plasmid pLP75. The XhoI/SalI fragment encoding SCG1 is transferred to the SalI site in pRS424 (Stratagene), forming plasmid pLP86.

EXAMPLE 6

Mutations in SST2 enhance the sensitivity of the responses to somatostatin

Mutations in the SST2 gene result in supersensitivity of otherwise wild type cells to mating pheromone. The effect of this mutation on levels of AT resistance expressed in far1, FUS1-HIS3 strains is examined (Figure 16). Cells (5 x 10^5) from overnight cultures of LY230 [a derivative of YPH50 (Statagene) MATa SST2 ura3-52 lys2-801 ade2 trp1Δ63 his3Δ200 leu2Δ1 far1ΔFUS1-HIS3] and LY238 (a modification of LY230 sst2ΔADE2) in SCD-ura, trp are plated on SCD-ura, trp, his, containing 10 mM AT and incubated at 30°C for 2 days. Increased AT resistance in

response to mating factor is exhibited by LY238 (sst2). Growth of LY238 is observed around disks containing 10 pmol of mating pheromone, while LY230 (SST2) required 1 nmol of mating factor for significant growth to be observed. Thus, introduction of sst2 into these strains raises the sensitivity of the bioassay by at least 100-fold, opening the possibility of increasing the sensitivity of other G protein-coupled receptor bioassays as well.

Yeast strains that express SSTR2 and bearing a defective sst2 gene exhibit much greater growth around disks containing various concentrations of somatostatin than is exhibited by strains containing a functional SST2 gene. Overnight cultures of strains LY268 (sst2, containing pLP82), LY266 (sst2, containing pLP83), LY288 [a derivative of YPH500 (Stratagene) MATa SST2 ura3-52 lys2-801 ade2 trp1Δ63 his3Δ200 leu2Δ1 far1ΔLYS2 scg1ΔhisG fus1ΔFUS1-HIS3 bearing the SSTR2 expression plasmid and the SCG1-Gα_{12} expression plasmid, pLP82] and LY290 (a modification of LY288 that contains pLP83) in SC Dextrose (2%) lacking ura, trp were transferred to SC Lactate medium (2%) lacking ura and trp, and subsequently to SC Galactose (2%) medium lacking ura and trp. Cells (2 x 10^5) are then plated in 30 ml SC Galactose (2%) plates lacking ura, trp and his, the indicated amounts of selected compounds applied to paper disks situated on the surface of the agar plate, and incubated at 30°C for 3-5 days. Halos of growth are observed around disks saturated with varying concentrations of S-14 in both sst2Δ and SST2 strains (Figure 17), however, the amount of growth exhibited by strain LY268 and LY266 (sst2) is significantly greater than that observed for strains LY288 and LY290 (SST2). Furthermore, the down-regulatory effect of SST2 is more pronounced in those strains that express solely the wild type SCG1/GPA1 protein, consistent with the expectation that SST2 interacts more faithfully with the native protein than the SCG1-Gα_{12}

chimeric protein.

EXAMPLE 7

Functional expression of a rat cholecystokinin (CCK_B) receptor in yeast.

Cholecystokinin (CCK) is a major intestinal hormone that plays an important role in regulating pancreatic secretion and bile ejection (1). CCK is also one of the most widely distributed of brain neuropeptides (2). CCK promotes its effects through the action of cell surface receptors which can be classified using pharmacological criteria into two subtypes, CCK_A and CCK_B (3). Molecular cloning efforts have identified cDNAs encoding G protein-coupled CCK_A (4) and CCK_B (5-8) receptors. Recently, compounds with selective CCK_B receptor antagonist properties having potent anxiolytic activity have been identified (9). Functional expression of CCK_B receptors in yeast should permit rapid screening for new compounds with CCK_B antagonist properties and facilitate molecular characterization of structural aspects of the CCK_B receptor required for rational design of new CCK_B ligands.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (10). The rat CCK_B receptor was cloned from rat brain cDNA by PCR using oligonucleotide primers that introduce BglII sites at 5' and 3' ends (5-AAAAGATCTAAAATGGACCTGCTCAAGCTG, 3' AAAAGATCTTCAGCCAGG CCCCAGTGTGCT). The CCK_B receptor expression plasmid, pJH20,

was constructed by inserting the BglII-digested PCR fragment in the correct orientation into BamHI cut pMP3 (11). The Gα protein expression plasmids used in this study were constructed by replacing DNA sequences encoding the 47 carboxy-terminal amino acids of GPA1 in pLP83 (11) with those of $G\alpha_s$(pLP122), $G\alpha_{12}$ (pLP121).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (12). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequential insertional deletion using recombinant alleles. Yeast strains that express the rat CCK_B receptor were constructed by sequential DNA-mediated transformation of LY296 (MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2, ref. 7) with pJH20 followed by the Gα protein expression plasmids described above.

Radiolabeled agonist saturation binding assays. Crude yeast membrane extracts from late log phase cultures were prepared by glass-bead lysis and centrifugation at 40,000 xg following a published procedure (13). The protein content of crude membrane fractions was measured using the Biorad protein assay kit according to manufacturers instructions. Radioligand binding assays were conducted according to Strnad et al (14) using ^{3}H-CCK-8 (Amersham) in the presence of 150 mM NaCl. Non-specific binding was defined as that observed in the presence of 1 μM CCK-4. Negligible specific binding was observed in membrane fractions made from cells lacking CCK_B receptor (data not shown).

Bioassay. Functional assay of CCK_B receptor expressed in yeast was accomplished using modification of a standard procedure (11). Yeast strains were grown overnight

in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and tryptophan (SCD-ura-trp) medium, washed to remove residual glucose and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (50°C) SC Galactose (2 %)-ura-trp-his agar medium (30 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 x10^4 cells/ml) and poured into square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of DMSO containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Cholecystokinins (CCK-4, CCK-8), somatostatin (S-14), and met-enkephalin were from Bachem. Oxymetazoline, isoproterenol, and carbachol were from Sigma.

RESULTS

Cholecystokinin binding to the rat CCK_B receptor expressed in yeast. High level functional expression of the rat CCK_B receptor in yeast was a necessary prerequisite to the development of a useful bioassay. The rat CCK_B receptor cDNA was placed under the control of the GAL1 promoter in plasmid pJH20. This construct also confers inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking GAL4 sequences (data not shown). CCK_B receptor sequences were introduced into pJH20 without modification of the protein coding sequences. Previously, King et al. reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent STE2 sequence was necessary for efficient receptor expression in yeast (15). In contrast, functional expression of CCK_B

receptor in yeast does not require addition of any yeast sequences to the amino-terminus. Plasmids conferring expression of chimeric Gα proteins composed of amino-terminal βγ-interaction domain from Gpa1p and carboxy-terminal receptor interaction domains from rat $G\alpha_{i2}$ (pLP121) or $G\alpha_s$ (pLP122) under the control of the *GPA1* promoter were constructed. Yeast strains that contain expressed CCK_B receptor and chimeric $G\alpha_{i2}$ (LY628) and $G\alpha_s$ (LY631) protein were assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with CCK_B receptor and Gα protein expression plasmids. Most G protein-coupled receptors exhibit both high and low agonist-dependent affinity states. High-affinity agonist binding is dependent on functional association of receptor with a heterotrimeric G protein. If the receptor does not associate with, or is uncoupled from the G protein, agonist binding will be of low affinity and undetectable in radiolabeled agonist saturation binding assays. In crude yeast membrane fractions made from LY631 cells, the agonist ^{3}H-CCK-8 bound to the CCK_B receptor with high affinity and in a saturable manner (Fig. 18), demonstrating that (1) a functional ligand-binding conformation of the CCK_B receptor was expressed in yeast, and (2) the receptor functionally associated with the chimeric Gα protein, resulting in a high-affinity agonist binding state. The CCK_B receptor expressed in yeast displayed an affinity for ^{3}H-CCK-8 (K_d = 8 nM) substantially lower than the high affinity binding state of the CCK_B receptor expressed in mammalian cells (K_d = 100 pM, ref. 5), perhaps due to an inefficient interaction with the receptor interaction domain from rat $G\alpha_s$ These binding parameters would be expected to more closely resemble the native values in extracts from cells containing the cognate Gα protein, Gαq (5-8). The total number of ^{3}H-CCK-8 binding sites observed (B_{max}=206 fmol/mg) was consistent with values

obtained for the yeast α-mating pheromone receptor (200 fmol/mg, ref. 16). For many G protein-coupled receptors, high affinity agonist binding is sensitive to GTP and its analogs. GTP analogs induce dissociation of the receptor/G-protein complex, resulting in a low affinity agonist binding state. Addition of GppNHp (100 μM), a non-hydrolyzable GTP analog, to an agonist binding assay decreased specific binding of ^{3}H-CCK-8 to the CCK_B receptor by greater than 50% in crude membrane fractions from LY631 cells. These results represent a further indication of functional coupling between the CCK_B receptor and the chimeric Gα protein. Thus, the rat CCK_B receptor expressed in yeast exhibits high-affinity agonist binding properties comparable to those observed in mammalian tissues.

The CCK_B receptor retained agonist selectivity when expressed in yeast. A selective and sensitive bioassay was designed using yeast strains bearing the above described genetic modifications and plasmids conferring expression of the CCK_B receptor and chimeric $G_{\alpha i2}$ and $G_{\alpha s}$ proteins. A dose-dependent growth response of LY628 and LY631 cells was evident around applied CCK-4 (Fig. 19). The assay was selective: the diameter of the growth zones was proportional to the reported affinity of the ligands for the CCK_B receptor (CCK-8 > gastrin > CCK-4) reflecting the ability of the bioassay to discriminate between ligands of varying potency (5-8). Detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (somatostatin, met-enkephalin, oxymetazoline, isoproterenol, carbachol), nor by yeast cells lacking the CCK_B receptor (data not shown). A detectable response was observed to 10 μg of CCK-4.

DISCUSSION

Compounds that act at the CCK receptors, particularly

antagonists, may possess great therapeutic potential (3). In the periphery, the inhibitory effects of CCK antagonists make them excellent candidates for treatment of pancreatitis, pancreatic cancer, biliary colic, disorders of gastric emptying, and irritable bowel syndrome. CCK antagonists reverse the development of satiety and might be useful in improving appetite in anorectic patients or others that require increased food intake. Conversely, CCK agonists might be useful appetite suppressants. CCK antagonists also potentiate opiate analgesia and so might be appropriate for use in the management of clinical pain. In the CNS, selective CCK antagonists have promise as powerful anxiolytic agents (9). Further, CCK antagonists relieve the anxiety associated with withdrawal from drug use, and so might find a use in the treatment of withdrawal from commonly abused drugs. CCK agonists may have use as antipsychotic agents.

REFERENCES CITED IN THIS EXAMPLE

1. **Ivy, A.C. and E. Oldberg.** 1928. J. Physiol. (London) **86**: 599-613.
2. **Dockray, G.J.R.** 1976. Immunochemical evidence of cholecystokinin-like peptides in brain. Nature **264**: 568-570.
3. **Woodruff, G.N. and J. Hughes.** 1991. Cholecystokinin antagonists. Annu. Rev. Pharmacol. Toxicol. **31**: 469-501.
4. **Wank, S.A, R. Harkins, R.T.Jensen, H. Shapiro, A. de Weerth, and T. Slattery.** 1992. Purification, molecular cloning, and function expression of the cholecystokinin receptor from rat pancreas. Proc. Natl. Acad. Sci. USA **89**: 3125-3129.
5. **Kopin, A.S., Y-M. Lee, E.W. McBride, L.J. Miller, M. Liu, H.Y. Lin, L.F. Kolakowski, and M. Beinborn.** 1992.

Expression cloning and characterization of the canine parietal cell gastrin receptor. Proc. Natl. Acad. Sci. USA **89**: 3605-3609.

6. **Wank, S.A., J.R. Pisenga, and A. de Weerth.** 1992. Brain and gastrointestinal cholecystokinin receptor family: Structure and function. Proc. Natl. Acad. Sci. USA **89**: 8691-8695.

7. **Lee, Y-M., M. Beinborn, E.W. McBride, M. Lu, L.F. Kolakowski. and A.S. Kopin** 1993. The human brain cholecystokinin-B/gastrin Receptor. J. Biol. Chem. **268**: 8164-8169.

8. **Ito, M., T. Matsui, T. Taniguchi, T. Tsukamoto, T. Murayama, N. Arima, H. Nakata, T. Chiba, and K. Chibura.** 1993. Functional characterization of a human brain cholecystokinin-B receptor. J. Biol. Chem. **268**: 18300-18305.

9. **Hughes, J., P. Boden, B. Costall, A. Domeney, E. Kelly, D.C. Horwell, J.C. Hunter, R.D. Pinnock, and G.N. Woodruff.** 1990. Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity. Proc. Natl. Acad. Sci. USA **87**: 6728-6732.

10. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning, A Laboratory Handbook. Cold Spring Harbor Press. Cold Spring Harbor, NY.

11. **Price, L.A., E.M. Kajkowski, J.R. Hadcock, B.A. Ozenberger, and M.H. Pausch.** 1995. Yeast cell growth in response to agonist dependent activation of a mammalian somatostatin receptor. submitted.

12. **Rose, M., F. Winston, and P. Hieter.** 1990. Methods in Yeast Genetics. Cold Spring Harbor Press. Cold Spring Harbor, NY.

13. **Blumer, K. J., J. E. Reneke, and J. Thorner.** 1988. The STE2 gene product is the ligand-binding component of the α-factor receptor of Saccharomyces cerevisiae.

J. Biol. Chem. **263**: 10836-10842.

14. **Strnad, J., C. M. Eppler, M. Corbett, and J. R. Hadcock.** 1993. The rat SSTR2 somatostatin receptor subtype is coupled to inhibition of cyclic AMP accumulation. Biochem. Biophys. Res. Comm. **191**: 968-976.

15. **King, K., H. G. Dohlman, J. Thorner, M. G. Caron, and R.J. Lefkowitz.** 1990. Control of yeast mating signal transduction by a mammalian β_2-adrenergic receptor and G_s α subunit. Science **250**: 121-123.

16. **Weiner, J. L., C. Guttierez-Steil, and K. J. Blumer.** 1993. Disruption of receptor-G protein coupling in yeast promotes the function of an SST2-dependent adaptation pathway. J. Biol. Chem. **268**: 8070-8077.

EXAMPLE 9

Functional expression of a rat adenosine (A_{2a}) receptor in yeast.

Adenosine, as well as ATP and related purinergic compounds, function as both neurohormonal agents and autocoids regulating the process of cell to cell communication (1). In this role, adenosine regulates a broad range of physiological functions including heart rate and contractility, smooth muscle tone, sedation, release of neurotransmitters, platelet function, lipolysis, kidney and white blood cell action. Adenosine promotes its effects through the action of cell surface receptors which can be classified using pharmacological criteria into three subtypes, A_1, A_{2a} and A_{2b}, and A_3. Molecular cloning efforts have identified cDNAs encoding G protein-coupled adenosine A_1 (2-5), A_{2a} and A_{2b} (6-9), and A_3 receptors (10). Functional expression of adenosine receptors in yeast should permit

rapid screening for new compounds with adenosine agonist and antagonist properties and facilitate molecular characterization of structural aspects of the adenosine receptors required for rational design of new adenosine ligands.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (11). The rat A_{2a}-adenosine receptor (9) was cloned from rat brain cDNA by PCR using oligonucleotide primers that introduce BamHI sites at 5' and 3' ends (5' GAAGATCTAAAAAATGGGCTCCTCGGTGTAC, 3' ACATGCATGCAGATCTTCAGGAAGGGGCAAACTC). The A_{2a}-adenosine receptor expression plasmid, pJH21, was constructed by inserting the BglII-digested PCR fragment in the correct orientation into BamHI cut pMP3 (12). For constitutive expression of the A_{2a}-adenosine receptor in glucose-containing medium, the expression vector, pLP100, was constructed. DNA fragments encoding transcriptional regulatory sequences from the *ADH1* gene (&) were amplified by PCR and inserted into pRS426. An ADH1 transcriptional terminator fragment was amplified from yeast genomic DNA (YPH501, Stratagene) using synthetic oligonucleotides that add 5' XhoI (TTTCTCGAGCGAATTTCTTATGATTT) and 3' KpnI (TTTGGTACCGGGCCCGGACGGATTACAACAGGT) sites. An *ADH1* promoter fragment was amplified from yeast genomic DNA using synthetic oligonucleotides that add 5' SacI GGGAGCTCTGATGGTGGTACATAACG) and 3' BamHI (GGGGGATCCTGTATATGAGATAGTTGA) sites. The A_{2a}-adenosine receptor expression plasmid, pLP116, was constructed by inserting a PCR fragment encoding the A_{2a}-adenosine receptor amplified using oligonucleotides that add 5' BglII (AAAGATCTAAAATGGGCTCCTCGGTGTAC) and 3' SalI (AAGTCGACTCAGGAA

GGGGCAAACTC) sites BamHI-SalI cut LP100. The Gα protein expression plasmids used in this study were constructed by replacing DNA sequences encoding the 47 carboxy-terminal amino acids of *GPA1* in pLP83 (12) with those of $G_{\alpha s}$ (pLP122) and $G\alpha_{i2}$ (pLP121).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (13). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequentional insertional deletion using recombinant alleles. Yeast strains that express the rat A_{2a}-adenosine receptor were constructed by sequential DNA-mediated transformation of LY296 (*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔAΔE2* , ref. 12) with A_{2a}-adenosine receptor expression plasmids followed by the Gα protein expression plasmids described above.

Radiolabeled agonist saturation binding assays. Crude yeast membrane extracts from late log phase cultures were prepared by glass-bead lysis and centrifugation at 40,000 xg following a published procedure (14). The protein content of crude membrane fractions was measured using the Biorad protein assay kit according to manufacturers instructions. Radioligand binding assays were conducted according to Strnad *et al.* (15) using ^{3}H-NECA (Amersham). Non-specific binding was defined as that observed in the presence of 1 μM NECA. Negligible specific binding was observed in membrane fractions made from cells lacking A_{2a}-adenosine receptor (data not shown).

Bioassay. Functional assay of the A_{2a}-adenosine receptor expressed in yeast was accomplished using a modification of a standard procedure (12). Yeast strains were grown overnight in 2 ml synthetic complete liquid

medium containing glucose (2 %) and lacking uracil and tryptophan (SCD-ura-trp) medium, washed to remove residual glucose and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (50°C) SC Galactose (2 %)-ura-trp-his agar medium (30 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) containing 5 mM 3-aminotriazole (Sigma) was inoculated with the overnight culture (2 $x10^4$ cells/ml) and plated in square (9 x 9 cm) petri plates. For expression of the A_{2a}-adenosine receptor in glucose-containing medium, samples were removed from the first overnight culture and assayed in agar medium composed as above with glucose (2%) replacing galactose. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of DMSO containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Adenosine ligands CGS-21680, NECA, and DPMA were purchased from RBI. Somatostatin (S-14) and met-enkephalin were from Bachem. Oxymetazoline, isoproterenol, and carbachol were from Sigma.

RESULTS

Adenosine agonist binding to the rat A_{2a}-adenosine receptor expressed in yeast. High level functional expression of the A_{2a}-adenosine receptor in yeast was a necessary prerequisite to the development of a useful bioassay. In plasmid pJH21, the rat A_{2a}-adenosine receptor cDNA was placed under the control of the inducible *GAL1* promoter. This construct also confers inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes; resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). Plasmid pLP116 confers high level constitutive expression of the A_{2a}-

adenosine receptor under the control of the *ADH1* promoter. In both plasmids, DNA sequences encoding the A_{2a}-adenosine receptor were introduced without modification of the protein coding sequences. Previously, King *et al.* reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient receptor expression in yeast (16). In contrast, functional expression of the A_{2a}-adenosine receptor in yeast does not require addition of any yeast sequences to the amino-terminus. A chimeric Gα protein composed of the proposed amino-terminal $\beta\gamma$-interaction domain from Gpalp and a carboxy-terminal receptor interaction domain from rat $G\alpha_s$ (pLP122) under the control of the *GPA1* promoter was constructed. Yeast strains that contain expressed A_{2a}-adenosine receptor and chimeric Gα protein were assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with A_{2a}-adenosine receptor and Gα protein expression plasmids.

Most G protein-coupled receptors exhibit both high and low agonist-dependent affinity states. High-affinity agonist binding is dependent on functional association of receptor with a heterotrimeric G protein. If the receptor does not associate with, or is uncoupled from the G protein, agonist binding will be of low affinity and undetectable in radiolabeled agonist saturation binding assays. In crude yeast membrane fractions from cells bearing pLP116 and pLP122 (LY626), the agonist ^{3}H-NECA bound to the A_{2a}-adenosine receptor with high affinity and in a saturable manner, (Fig. 20) demonstrating that (1) a functional ligand-binding conformation of the A_{2a}-adenosine receptor was expressed in yeast, and (2) the receptor functionally associated with the chimeric Gα protein, resulting in a high-affinity agonist binding state. The total number of ^{3}H-

NECA binding sites observed (B_{max}=242 fmol/mg) exceeded values obtained for the yeast α-mating pheromone receptor (200 fmol/mg, ref. 17). The affinity of [^{3}H] NECA for the A_{2a}-adenosine receptor in yeast membranes (Kd-8μM) is equivalent to that observed in mammalian cells indicating functional coupling between receptor and G protein.

The A_{2a}-adenosine receptor retained agonist selectivity when expressed in yeast. A selective and sensitive bioassay was designed using a yeast strain (LY595) bearing the above described genetic modifications and plasmids conferring expression of the A_{2a}-adenosine receptor (pLP116) and *GPA1* (pLP83). A dose-dependent growth response of LY595 cells was evident around applied CGS-21680, an A_{2a}-adenosine receptor selective agonist. The growth response was significantly more robust than that exhibited by cells responding to NECA and DPMA (Fig. 21). The assay was selective: the diameter of the growth zones was proportional to the reported affinity of the ligands for the A_{2a}-adenosine receptor (CGS-21680>NECA=DPMA) reflecting the ability of the bioassay to discriminate between ligands of varying potency. Detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (somatostatin, met-enkephalin, oxymetazoline, isoproterenol, carbachol), nor by yeast cells lacking the A_{2a}-adenosine receptor (data not shown). A detectable response was observed to 10 μg of CGS-21680.

DISCUSSION

Multiple therapeutic opportunities exist for compounds that modulate the function of the adenosine receptors (1). Adenosine agonists may be useful in the treatment of epileptic seizure episodes and in preventing neuronal damage in stroke and neurodegenerative disorders. The antidysrhythmic action adenosine suggests that adenosine

agonists could be effective in the treatment of complex tachycardia. A_2 adenosine agonists have potent sedative, anticonvulsant and anxiolytic activity. A_{2a}-adenosine selective agonists may be useful in stimulating lipolysis in adipose tissue, making them useful as weight loss treatments or antidiabetic agents and in the improvement of carcass quality in agricultural animals. A_1 adenosine antagonists may be useful in treatment of acute renal dysfunction. A_3-antagonists may be useful in modulating mast cell degranulation for treatment of inflamatory disorders, including asthema.

REFERENCES CITED IN THIS EXAMPLE

1. **Jacobson, K.A., P.J.M. van Galen, and M. Williams.** 1992. Adenosine receptors: Pharmacology, structure-activity relationships, and therapeutic potential.J. Med. Chem. **35**: 407-422.
2. **Libert, F., S.N. Schiffmann, A. Lefort, M. Parmentier, C. Gerard, J.E. Dumont, J.-J. Vanderhaeghen, and G. Vassart.** 1991. The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor. EMBO J. **10**: 1677-1682.
3. **Mahan, L.C., L.D. McVittie, E.M. Smyk-Randal, H. Nakata, F.J. Monsma, C.R. Green, and D.R. Sibley.** 1991. Cloning and expression of an A_1 adenosine receptor from rat brain. Mol. Pharmocol. **40**: 1-7.
4. **Olah, M.E., H. Ren, J. Ostrowski, K.A. Jacobson, and G.L. Stiles.** 1992. Cloning, expression and characterization of the unique bovine A_1 adenosine receptor. J. Biol. Chem. **267**: 10764-10770.
5. **Tucker, A.L., J. Linden, A.S. Robeva, D.D. D'angelo, and K.R. Lynch.** 1992. Cloning and expression of a bovine adenosine A_1 receptor cDNA. FEBS Lett. **297**: 107-111.
6. **Maenhaut, C., J. van Sande, F. Libert, M. Abramowicz, M. Parmetier, J.-J. E. Dumont, G.**

Vassart, and S Schiffmann. 1990. RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. Bioch. Biophys. Res. Comm. **173:** 1169-1178.

7. **Pierce, K.D., Furlong, T.J., L.A. Selbie, and J. Shine.** 1992. Molecular cloning and expression of an adenosine A2b receptor from human brain. Bioch. Biophys. Res. Comm. **187:** 86-93.

8. **Chern, Y., K. King, H.-L. Lai, and H.-T. Lai.** 1992. Molecular cloning of a novel adenosine receptor gene from rat brain. Bioch. Biophys. Res. Comm. **185:** 304-309.

9. **Stehle, J.H., S.A. Rivkees, J.J. Lee, D.R. Weaver, J.D. Deeds, and S.M. Reppert.** 1992. Molecular cloning and expression of the cDNA for a novel A_2 adenosine receptor subtype. Mol. Endocrinol. **6:** 384-393.

10. **Zhou, Q.-Y., C.Li, M.E. Olah, R.A. Johnson, G.L.Stiles, and O. Civelli.** 1992. Molecular cloning and characterization of an adenosine receptor: The A3 adenosine receptor. Proc. Natl. Acad. SCI. USA. **89:** 7432-7436.

11. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning, A Laboratory Handbook.* Cold Spring Harbor Press. Cold Spring Harbor, NY.

12. **Price, L.A., E.M. Kajkowski, J.R. Hadcock, B.A. Ozenberger, and M.H. Pausch.** 1995. Yeast cell growth in response to agonist dependent activation of a mammalian somatostatin receptor. submitted.

13. **Rose, M., F. Winston, and P. Hieter.** 1990. *Methods in Yeast Genetics.* Cold Spring Harbor Press. Cold Spring Harbor, NY.

14. **Blumer, K. J., J. E. Reneke, and J. Thorner.** 1988. The *STE2* gene product is the ligand-binding component of the α-factor receptor of *Saccharomyces cerevisiae.* J. Biol. Chem. **263:** 10836-10842.

15. **Strnad, J., C. M. Eppler, M. Corbett, and J. R.**

Hadcock. 1993. The rat SSTR2 somatostatin receptor subtype is coupled to inhibition of cyclic AMP accumulation. Biochem. Biophys. Res. Comm. **191**: 968-976.

16. **King, K., H. G. Dohlman, J. Thorner, M. G. Caron, and R.J. Lefkowitz**. 1990. Control of yeast mating signal transduction by a mammalian β_2-adrenergic receptor and G_s α subunit. Science **250**: 121-123.

17. **Weiner, J. L., C. Guttierez-Steil, and K. J. Blumer**. 1993. Disruption of receptor-G protein coupling in yeast promotes the function of an *SST2*-dependent adaptation pathway. J. Biol. Chem. **268**: 8070-8077.

EXAMPLE 8

Functional expression of a rat somatostatin subtype 5 receptor in yeast.

The cyclic tetradecapeptide somatostatin is a potent inhibitor of secretion of several hormones, including growth hormone from the pituitary, glucagon and insulin from the pancreas, and gastrin from the gut. Somatostatin also acts as a neurotransmitter and has been shown to have broad modulatory effects in CNS and peripheral tissues (1). The effects of somatostatin are transduced through binding of the hormone to high-affinity, plasma membrane localized somatostatin (SSTR) receptors (2). The SSTR's, encoded in five distinct subtypes (SSTR1-5), which account in part for tissue-specific differences in responses to somatostatin (3-10), comprise a subfamily of the seven-transmembrane domain, G protein-coupled receptor superfamily that mediates responses to a broad variety of extracellular signals. Functional expression of SSTR5 in yeast should permit rapid screening for new subtype-selective somatostatin

agonists and compounds with antagonist properties and facilitate molecular characterization of structural aspects of the SSTR5 required for rational design of new somatostatin ligands.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (11). The rat SSTR5 (7) was cloned from rat genomic DNA by PCR using oligonucleotide primers that introduce BglII sites at 5' and 3' ends (5'AAAAAGATCTAAAATGGAGCCCCTCTCTCTG, 3' AGCAGATCTTCAGATC CCAGAAGACAAC). The SSTR5 expression plasmid, pJH19, was constructed by inserting the BglII-digested PCR fragment in the correct orientation into BamHI cut pMP3 (12). The Gα protein expression plasmids used in this study were constructed by replacing DNA sequences encoding the 47 carboxy-terminal amino acids of *GPA1* in pLP83 (12) with those of $G\alpha_s$ (pLP122), $G\alpha_{i2}$ (pLP121).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (13). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequential insertional deletion using recombinant alleles. Yeast strains that express SSTR5 were constructed by sequential DNA-mediated transformation of LY296 (*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2*, ref. 12) with pJH19 followed by the Gα protein expression plasmids described above.

Bioassay. Functional assay of SSTR5 expressed in yeast

was accomplished using modification of a standard procedure (12). Yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and tryptophan (SCD-ura-trp) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (55 °C) SC Galactose (2 %)-ura-trp-his agar medium (30 µl, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 x10^4 cells/ml) and plated in square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Somatostatin (S-14, S-28), met-enkephalin, and CCK-8 were from Bachem. Oxymetazoline, isoproterenol, and carbachol were from Sigma.

RESULTS

Somatostatin dependent growth response of yeast cells expressing the SSTR5. High level functional expression of the SSTR5 in yeast was a necessary prerequisite to the development of a useful bioassay. The SSTR5 cDNA was placed under the control of the *GAL1* promoter in plasmid pJH19. This construct also confers inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). SSTR5 sequences were introduced into pJH19 without modification of the protein coding sequences. Previously, King *et al.* reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient

receptor expression in yeast (15). In contrast, functional expression of SSTR5 in yeast does not require addition of any yeast sequences to the amino-terminus. A chimeric Gα protein composed of the proposed amino-terminal β_2-interaction domain from Gpalp and a carboxy-terminal receptor interaction domain from rat $G\alpha_{12}$ (pLP121) under the control of the *GPA1* promoter was constructed. A yeast strain (LY620) that contains expressed SSTR5 and chimeric Gα protein was assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with SSTR5 (pJH19) and Gα protein expression (pLP121) plasmids. A dose-dependent growth response of LY620 cells was evident around applied S-14 (Fig. 22). Detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (CCK-8, met-enkephalin, oxymetazoline, isoproteranol, carbachol), nor by yeast cells lacking the SSTR5 (data not shown). A detectable response was observed to 60 nmol of S-14.

REFERENCES CITED IN THIS EXAMPLE

1. **Brazeau, P., W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier and R. Guillemin.** 1973. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science **129**: 77-79.
2. **Reisine, T. and G. I. Bell** 1993. Molecular biology of somatostatin receptors. Trends Neurosci. **16**: 34-38.
3. **Meyerhof, W., H.-J. Paust, C. Schonrock, and D. Richter.** 1991. Cloning of a cDNA encoding a novel putative G protein-coupled receptor expressed in specific rat brain regions. DNA Cell Biol. **10**:

689-694.

4. **Bruno, J. F., Y. Xu, J. Song, and M. Berelowitz.** 1992. Molecular cloning and functional expression of a brain specific somatostatin receptor. Proc. Natl. Acad. Sci. USA **89**: 11151-11155.

5. **Kluxen, F.-W., C. Bruns, and H. Lubbert.** 1992. Expression cloning of a rat brain somatostatin receptor. Proc. Natl. Acad. Sci. USA **89**: 4618-4622.

6. **Li, X.-J., M. Forte, R. A. North, C. A. Ross, and S. H. Snyder.** 1992. Cloning and expression of a rat somatostatin receptor enriched in brain. J. Biol. Chem. **267**: 21307-21312.

7. **O'Carrol, A.-M., S. J. Lolait, M. Konig, and L. Mahan.** 1992. Molecular cloning and expression of a pituitary somatostatin receptor with preferential affinity for somatostatin-28. *Mol. Pharmocol.* **42**: 939-946.

8. **Yasuda, K., S. Rens-Domiano, C. D. Breder, S. F. Law, C.B.Saper, T. Reisine and G. I. Bell.** 1992. Cloning of a novel somatostatin receptor, SSTR3, coupled to adenylylcyclase. J. Biol. Chem. **28**: 20422-20428.

9. **Yamada, Y., S. R. Post, K. Wang, H. S. Tager, G. I. Bell and S. Seino.** 1992. Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. Proc. Natl. Acad. Sci. USA **89**: 251-255.

10. **Strnad, J., C. M. Eppler, M. Corbett, and J. R. Hadcock.** 1993. The rat SSTR2 somatostatin receptor subtype is coupled to inhibition of cyclic AMP accumulation. Biochem. Biophys. Res. Comm. **191**: 968-976.

11. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning, A Laboratory Handbook.* Cold Spring Harbor Press. Cold Spring Harbor, NY.

12. **Price, L.A., E.M. Kajkowski, J.R. Hadcock, B.A. Ozenberger, and M.H. Pausch.** 1995. Yeast cell growth in response to agonist dependent activation of a mammalian somatostatin receptor. submitted.

13. **Rose, M., F. Winston, and P. Hieter.** 1990. *Methods in Yeast Genetics*. Cold Spring Harbor Press. Cold Spring Harbor, NY.

14. **Blumer, K. J., J. E. Reneke, and J. Thorner.** 1988. The *STE2* gene product is the ligand-binding component of the α-factor receptor of *Saccharomyces cerevisiae*. J. Biol. Chem. **263**: 10836-10842.

15. **King, K., H. G. Dohlman, J. Thorner, M. G. Caron, and R.J. Lefkowitz.** 1990. Control of yeast mating signal transduction by a mammalian β_2-adrenergic receptor and Gα α subunit. Science **250**: 121-123.

16. **Weiner, J. L., C. Guttierez-Steil, and K. J. Blumer.** 1993. Disruption of receptor-G protein coupling in yeast promotes the function of an *SST2*-dependent adaptation pathway. J. Biol. Chem. **268**: 8070-8077.

Example 10

Functional expression of the porcine somatostatin subtype 2 (SSTR2) receptor in yeast.

The cyclic tetradecapeptide somatostatin is a potent inhibitor of secretion of several hormones, including growth hormone from the pituitary, glucagon and insulin from the pancreas, and gastrin from the gut. Somatostatin also acts as a neurotransmitter and has been shown to have broad modulatory effects in CNS and peripheral tissues (1). The effects of somatostatin are transduced through binding of the hormone to high-affinity, plasma membrane localized somatostatin (SSTR) receptors (2). The SSTR's, encoded in five distinct

subtypes (SSTR1-5), which account in part for tissue-specific differences in responses to somatostatin (3-11), comprise a subfamily of the seven-transmembrane domain, G protein-coupled receptor superfamily that mediates responses to a broad variety of extracellular signals. Functional expression of porcine SSTR2 in yeast should permit rapid screening for new species and subtype-selective somatostatin agonists and compounds with antagonist properties and facilitate molecular characterization of structural aspects of the porcine SSTR2 required for rational design of new somatostatin ligands. Compounds identified in high-throughput, mechanism-based screens represent leads for new growth-enhancing agents for use in pigs.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (12). The porcine SSTR2 (11) was cloned from a human brain cDNA library by PCR using oligonucleotide primers that introduce BglII sites at 5' and 3' ends (5' AAAAGATCTAAAATGTCCATTCCATTTGAC, 3' AAAAGGTACCAGATCTTCAGATACTGGTTTGGAG). The porcine SSTR2 expression plasmid, pJH18, was constructed by inserting the BglII-digested PCR fragment in the correct orientation into BamHI cut pMP3 (13).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (14). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequential insertional deletion using recombinant alleles. Yeast strains that express porcine SSTR2 were constructed by sequential DNA-mediated

transformation of LY296 (*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2* , ref. 13) with the chimeric G_{a12} protein expression plasmid, pLP82 (13), followed by pJH18 or pJH17.

Radiolabeled agonist saturation binding assays. Crude yeast membrane extracts from late log phase cultures were prepared by glass-bead lysis and centrifugation at 40,000 xg following a published procedure (15). The protein content of crude membrane fractions was measured using the Biorad protein assay kit according to manufacturers instructions. Radioligand binding assays were conducted according to Strnad *et al* (10) using radiolabeled somatostatin (^{125}I-tyr^{11}-S-14, Amersham). Non-specific binding was defined as that observed in the presence of 1 μM S-14. Negligible specific binding was observed in membrane fractions made from cells lacking porcine SSTR2 (data not shown).

Bioassay. Functional assay of the porcine SSTR2 expressed in yeast was accomplished using modification of a standard procedure (13). Yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and tryptophan (SCD-ura-trp) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (55 °C) SC Galactose (2 %)-ura-trp-his agar medium (30 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 x10^4 cells/ml) and plated in square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 μl of sterile water containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Somatostatin (S-14, S-28), met-enkephalin were from Bachem. Oxymetazoline, isoproterenol, and carbachol

were from Sigma. MK678 and sandostatin were prepared synthetically.

RESULTS

Somatostatin binding to the porcine SSTR2 expressed in yeast. High level functional expression of the porcine SSTR2 in yeast was a necessary prerequisite to the development of a useful bioassay. The porcine SSTR2 cDNA was placed under the control of the *GAL1* promoter in plasmid pJH17 and 18. These constructs also confer inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). The porcine SSTR2 sequences were introduced into pJH18 without modification of the protein coding sequences. Previously, King *et al.* reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient receptor expression in yeast (16). In contrast, functional expression of porcine SSTR2 in yeast does not require addition of any yeast sequences to the amino-terminus. A chimeric Gα protein composed of the proposed amino-terminal $\beta\gamma$-interaction domain from Gpa1p and a carboxy-terminal receptor interaction domain from rat $G\alpha_{12}$ (pLP82) under the control of the *GPA1* promoter was constructed. Yeast strains that contain expressed porcine SSTR2 and chimeric G_α protein were assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with porcine SSTR2 (pJH17, pJH18) and G_α protein expression (pLP82) plasmids.

Most G protein-coupled receptors exhibit both high and low agonist-dependent affinity states. High-

affinity agonist binding is dependent on functional association of receptor with a heterotrimeric G protein. If the receptor does not associate with, or is uncoupled from the G protein, agonist binding will be of low affinity and undetectable in radiolabeled agonist saturation binding assays. In crude yeast membrane fractions from cells bearing pJH17, the agonist ^{125}I-tyr^{11}-S-14 bound to the porcine SSTR2 with high affinity and in a saturable manner, demonstrating that (1) a functional ligand-binding conformation of the porcine SSTR2 was expressed in yeast, and (2) the receptor functionally associated with the chimeric Gα protein, resulting in a high-affinity agonist binding state. The total number of ^{125}I-tyr^{11}-S-14 binding sites observed (B_{max}=146 fmol/mg) was consistent with values obtained for the yeast α-mating pheromone receptor (200 fmol/mg, ref. 17).

The porcine SSTR2 retained agonist selectivity when expressed in yeast. A selective and sensitive bioassay was designed using a yeast strain (LY474) bearing the above described genetic modifications and plasmids conferring expression of the porcine SSTR2 (pJH18) and a Gpal-Gα_{i2} chimeric protein (pLP82). A dose-dependent growth response of LY474 cells was evident around applied S-14, MK678, and sandostatin (Fig. 23). The assay was selective: the diameter of the growth zones was proportional to the reported affinity of the ligands for the porcine SSTR2 (S-14=MK678>sandostatin) reflecting the ability of the bioassay to discriminate between ligands of varying potency (18). Detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (met-enkephalin, oxymetazoline, isoproterenol, carbachol), nor by yeast cells lacking the porcine SSTR2 (data not shown). A detectable response was observed to as little as 60 pmol of S-14, illustrating the exquisite sensitivity of the bioassay.

REFERENCES CITED IN THIS EXAMPLE

1. **Brazeau, P., W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier and R. Guillemin.** 1973. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science **129:** 77-79.
2. **Reisine, T. and G. I. Bell** 1993. Molecular biology of somatostatin receptors. Trends Neurosci. **16:** 34-38.
3. **Meyerhof, W., H.-J. Paust, C. Schonrock, and D. Richter.** 1991. Cloning of a cDNA encoding a novel putative G protein-coupled receptor expressed in specific rat brain regions. DNA Cell Biol. **10:** 689-694.
4. **Bruno, J. F., Y. Xu, J. Song, and M. Berelowitz.** 1992. Molecular cloning and functional expression of a brain specific somatostatin receptor. Proc. Natl. Acad. Sci. USA **89:** 11151-11155.
5. **Kluxen, F.-W., C. Bruns, and H. Lubbert.** 1992. Expression cloning of a rat brain somatostatin receptor. Proc. Natl. Acad. Sci. USA **89:** 4618-4622.
6. **Li, X.-J., M. Forte, R. A. North, C. A. Ross, and S. H. Snyder.** 1992. Cloning and expression of a rat somatostatin receptor enriched in brain. J. Biol. Chem. **267:** 21307-21312.
7. **O'Carrol, A.-M., S. J. Lolait, M. Konig, and L. Mahan.** 1992. Molecular cloning and expression of a pituitary somatostatin receptor with preferential affinity for somatostatin-28. *Mol. Pharmocol.* **42:** 939-946.
8. **Yasuda, K., S. Rens-Domiano, C. D. Breder, S. F. Law, C.B.Saper, T. Reisine and G. I. Bell.** 1992. Cloning of a novel somatostatin receptor, SSTR3, coupled to adenylylcyclase. J. Biol. Chem. **28:** 20422-20428.

9. **Yamada, Y., S. R. Post, K. Wang, H. S. Tager, G. I. Bell and S. Seino.** 1992. Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. Proc. Natl. Acad. Sci. USA **89**: 251-255.

10. **Strnad, J., C. M. Eppler, M. Corbett, and J. R. Hadcock.** 1993. The rat SSTR2 somatostatin receptor subtype is coupled to inhibition of cyclic AMP accumulation. Biochem. Biophys. Res. Comm. **191**: 968-976.

11. **Matsumoto, K., Y. Yokogoshi, Y. Fujinaka, C. Z. Xhang, and S. Saito.** 1994. Molecular cloning and sequencing of porcine somatostatin receptor 2. Biochem. Biophys. Res. Comm. **199**: 298-305.

12. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning, A Laboratory Handbook*. Cold Spring Harbor Press. Cold Spring Harbor, NY.

13. **Price, L.A., E.M. Kajkowski, J.R. Hadcock, B.A. Ozenberger, and M.H. Pausch.** 1995. Yeast cell growth in response to agonist dependent activation of a mammalian somatostatin receptor. submitted.

14. **Rose, M., F. Winston, and P. Hieter.** 1990. *Methods in Yeast Genetics*. Cold Spring Harbor Press. Cold Spring Harbor, NY.

15. **Blumer, K. J., J. E. Reneke, and J. Thorner.** 1988. The *STE2* gene product is the ligand-binding component of the α-factor receptor of *Saccharomyces cerevisiae*. J. Biol. Chem. **263**: 10836-10842.

16. **King, K., H. G. Dohlman, J. Thorner, M. G. Caron, and R.J. Lefkowitz.** 1990. Control of yeast mating signal transduction by a mammalian β_2-adrenergic receptor and G_s α subunit. Science **250**: 121-123.

17. **Weiner, J. L., C. Guttierez-Steil, and K. J. Blumer.** 1993. Disruption of receptor-G protein coupling in yeast promotes the function of an *SST2*-dependent adaptation pathway. J. Biol. Chem. **268**: 8070-

8077.

18. **Marbach, P., W. Bauer, and U. Briner.** 1988. Structure-function relationships of somatostatin analogs. Horm Res. **29**: 54-58.

EXAMPLE 11

Deletion of *MSG5* increases the sensitivity of response to agonist.

The responsiveness of a signal transduction system to a persistent stimulus diminishes with time. This phenomenon, known as desensitization or adaptation, is a universal characteristic of signal response systems. Several molecular mechanisms for adaptation have been described for the yeast mating signal transduction pathway (1). Mutations in the *SST2* gene confer defects in adaptation and increased mating pheromone sensitivity (2,3). The response to applied somatostatin by yeast cells that functionally express the rat SSTR2 is greatly increased in *sst2* mutant cells (4). Mutations in others genes whose products play a role in the adaptation response would be expected to have similar effects. Mutations in the *MSG5* gene, which encodes a putative protein tyrosine phosphatase, cause increased sensitivity to mating pheromone (5). In this study, deletion of *MSG5* in cells that express the rat SSTR2 greatly increases sensitivity to somatostatin. The effect of the *MSG5* mutation is additive with an *SST2* deletion mutation. The double mutant *sst2 msg5* cells form the basis of an extremely sensitive bioassay for compounds that interact with G protein-coupled receptors and G proteins.

MATERIALS AND METHODS

Strain constructions. All molecular biological

manipulations were performed according to standard procedures (6). Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (7). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used in these experiments were constructed using the recombinant *msg5ΔLEU2* allele in pS/PDel and multicopy YEp*MSG5* (5). Yeast strains bearing altered MSG5 levels were constructed by DNA-mediated transformation of LY268 (*MATa ura3-52 trp1D63 his3D200 leu2D1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2*, pJH2, pLP82) yielding MPY459 (LY268 *msg5ΔLEU2 sst2ΔADE2*) and MPY467 (LY268 YEp*MSG5*) and LY288 (LY268 *SST2*) yielding MPY458 (LY288 *msg5ΔLEU2 SST2*) and MPY466 (LY288 YEp*MSG5*) (4).

Bioassay. Functional bioassay of the rat SSTR2 expressed in yeast was accomplished using modification of a standard procedure (4). Yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil, tryptophan and leucine (SCD-ura-trp-leu) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp-leu liquid medium. Molten (55 °C) SC Galactose (2 %)-ura-trp-leu-his agar medium (35 ml, adjusted to pH 6.8 by addition of concentrated NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 x10^4 cells/ml) and plated in square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the somatostatin (S-14). Plates were incubated at 30 °C for 3 days. Somatostatin (S-14) was from Bachem.

RESULTS

Deletion of *MSG5* promotes increased sensitivity

to ligand. The effects of alterations in the expression of the *MSG5* gene product were assessed by comparing the growth response to S-14 by cells that express the rat SSTR2 (Fig. 24). Mutations that abolish *MSG5* adaptation function would be expected to increase the sensitivity of the bioassay to applied S-14, while overexpression of *MSG5* should blunt the growth response. As expected, a dose-dependent growth response to applied S-14 was observed for LY288 (*SST2 MSG5*). Consistent with expectations, deletion of the *MSG5* gene in MPY458 causes a substantial improvement in sensitivity of the bioassay. The growth response of MPY458 is comparable to that exhibited by LY268 (*sst2ΔADE2, MSG5*). The effects of mutations in both genes was observed in the double mutant MPY459 (*msg5ΔLEU2 sst2ΔADE2*) which showed a further improvement in the growth response. Overexpression of *MSG5* in *SST2* (MPY466) and *sst2ΔADE2* (MPY467) strains severely reduced the growth response.

REFERENCES CITED IN THIS EXAMPLE

1. **Sprague, G. F. and J. W. Thorner.** 1992. Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae*, 657-744. In J. R. Pringle, E.W. Jones and J.R. Broach, *Gene expression*, 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. **Chan, R. K. and C. A. Otte.** 1982. Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by α factor and a factor pheromones. Mol. Cell. Biol. **2**: 11-20.
3. **Chan, R. K. and C. A. Otte.** 1982. Physiological characterization of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by α factor and a factor pheromones. Mol. Cell. Biol. **2**: 21-29.
4. **Price, L.A., E.M. Kajkowski, J.R. Hadcock, B.A. Ozenberger, and M.H. Pausch.** 1995. Yeast cell

growth in response to agonist dependent activation of a mammalian somatostatin receptor. submitted.

5. **Doi, K., A. Gartner, G. Ammerer, B. Errede, H. Shinkawa, K. Sugimoto, and K. Matsumoto.** 1994. MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S. cerevisiae*. EMBO J. 13: 61-70.

6. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning, A Laboratory Handbook*. Cold Spring Harbor Press. Cold Spring Harbor, NY.

7. **Rose, M., F. Winston, and P. Hieter.** 1990. *Methods in Yeast Genetics*. Cold Spring Harbor Press. Cold Spring Harbor, NY.

EXAMPLE 12.

Functional expression of a human growth hormone release factor (GRF) receptor in yeast.

The growth hormone release factor (GRF) is a potent stimulator of secretion of growth hormone from the pituitary (1). The effects of GRF are transduced through binding of the hormone to high-affinity, plasma membrane localized GRF receptors. The GRF receptor and related secretin-class receptors comprise a subfamily of the seven-transmembrane domain, G protein-coupled receptor superfamily that mediates responses to a broad variety of extracellular signals, and are distinguished by the presence of a large amino-terminal ligand-binding domain (2). Functional expression of the human GRF receptor (3) in yeast should permit rapid screening for new species-selective agonists and facilitate molecular characterization of structural aspects of the GRF receptor required for rational design of new GRF receptor ligands. GRF agonists represent a new class of growth promoting agents for use in agricultural animals and may

find human therapeutic application in the management of growth of children of short stature.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (4). The human GRF receptor (3) was cloned from a human brain cDNA library by PCR using oligonucleotide primers that introduce BamHI sites at 5' and 3' ends (5' ATAGGATCCAAAATGGACCGCCGGATGTGGGGG, 3' ATATGGATCCCTAGCACATAGATGTCAG). The GRF receptor expression plasmid, pJH25, was constructed by inserting the BamHI-digested PCR fragment in the correct orientation into BamHI cut pMP3 (5). The G_a protein expression plasmids used in this study were constructed by replacing DNA sequences encoding the 47 carboxy-terminal amino acids of *GPA1* in pLP83 (12) with those of $G\alpha_s$ (pLP122).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (6). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequential insertional deletion using recombinant alleles. Yeast strains that express human GRF receptor were constructed by sequential DNA-mediated transformation of LY296 (*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2*, ref. 5) with pJH25, followed by the chimeric G_{as} protein expression plasmid, pLP122 (5).

Bioassay. Functional assay of the human GRF receptor expressed in yeast was accomplished using modification of a standard procedure (5). Yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and

tryptophan (SCD-ura-trp) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (55 °C) SC Galactose (2 %)-ura-trp-his agar medium (30 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 $x10^4$ cells/ml) and plated in square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Human GRF (hGRF (1-29)-NH_2), (D-ala^2)-hGRF (1-29)-NH_2, and met-enkephalin were from Bachem. Oxymetazoline, isoproterenol, and carbachol were from Sigma.

RESULTS

GRF binding to the human GRF receptor expressed in yeast. High level functional expression of the human GRF receptor in yeast was a necessary prerequisite to the development of a useful bioassay. The GRF receptor cDNA was placed under the control of the *GAL1* promoter in plasmid pJH25. These constructs also confer inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). The GRF receptor sequences were introduced into pJH25 without modification of the protein coding sequences. Previously, King *et al.* reported that replacement of the amino-terminal domain of the b_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient receptor expression in yeast (9). In contrast, functional expression of GRF receptor in yeast does not require addition of any yeast sequences to the amino-terminus. A chimeric Gα protein composed of the proposed

amino-terminal βγ-interaction domain from Gpa1p and a carboxy-terminal receptor interaction domain from rat $G_{\alpha s}$ (pLP122) under the control of the *GPA1* promoter was constructed. Yeast strains that contain expressed GRF receptor and chimeric G_{α} protein were assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with human GRF receptor (pJH25) and chimeric Gpa1-$G\alpha_s$ protein expression (pLP122) plasmids.

The human GRF receptor retained agonist selectivity when expressed in yeast. A selective and sensitive bioassay was designed using a yeast strain (CY990) bearing the above described genetic modifications and plasmids conferring expression of the human GRF receptor (pJH25) and a Gpa1-$G\alpha_s$ chimeric protein (pLP122). A dose-dependent growth response of CY990 cells was evident around an agonist analog of GRF [hGRF (1-29)-NH_2, Fig.25A] which was inhibited by coadministration of an antagonist analog [(D-arg^2)-hGRF (1-29)-NH_2, Fig. 25B]. The assay was selective: detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (met-enkephalin, oxymetazoline, isoproteranol, carbachol), nor by yeast cells lacking the GRF receptor (data not shown). A detectable response was observed to 20 nmol of GRF, illustrating the sensitivity of the bioassay.

REFERENCES CITED IN THIS EXAMPLE

1. **Bohlen, P. F. Esch, P. Brazeau, N. Ling, and Guillemin.** 1983. Isolation and characterization of the porcine hypothalamic growth hormone releasing factor. Biochem. Biophys. Res. Comm. **116**: 726-734.

2. **Segre, G.V. and S.R. Goldring.** 1993. Receptors for

secretin, calcitonin, parathyroid hormone (PTH)/PTH-related peptide, vasoactive intestinal peptide, glucagonlike peptide 1, growth hormone releasing hormone, and glucagon belong to a newly discovered G protein linked receptor family. Trends Endo. Metab. 4: 309-314.

3. **Gaylinn, B.D., J.K. Harrison, J.R. Zysk, C.E. Lyons, K.R. Lynch, and M.O. Thorner** 1993. Molecular cloning and expression of a human anterior pituitary receptor for growth hormone-releasing hormone. Mol. Endocrinol. **7**: 77-84.

4. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning, A Laboratory Handbook.* Cold Spring Harbor Press. Cold Spring Harbor, NY.

5. **Price, L.A., E.M. Kajkowski, J.R. Hadcock, B.A. Ozenberger, and M.H. Pausch.** 1995. Yeast cell growth in response to agonist dependent activation of a mammalian somatostatin receptor. submitted.

6. **Rose, M., F. Winston, and P. Hieter.** 1990. *Methods in Yeast Genetics.* Cold Spring Harbor Press. Cold Spring Harbor, NY.

7. **Blumer, K. J., J. E. Reneke, and J. Thorner.** 1988. The *STE2* gene product is the ligand-binding component of the α-factor receptor of *Saccharomyces cerevisiae.* J. Biol. Chem. **263**: 10836-10842.

8. **Strnad, J., C. M. Eppler, M. Corbett, and J. R. Hadcock.** 1993. The rat SSTR2 somatostatin receptor subtype is coupled to inhibition of cyclic AMP accumulation. Biochem. Biophys. Res. Comm. **191**: 968-976.

9. **King, K., H. G. Dohlman, J. Thorner, M. G. Caron, and R.J. Lefkowitz.** 1990. Control of yeast mating signal transduction by a mammalian β_2-adrenergic receptor and G_s α subunit. Science **250**: 121-123.

10. **Weiner, J. L., C. Guttierez-Steil, and K. J. Blumer.** 1993. Disruption of receptor-G protein coupling in yeast promotes the function of an *SST2*-dependent

adaptation pathway. J. Biol. Chem. **268**: 8070-8077.

EXAMPLE 13

Overexpression of *STE50* enhances the sensitivity of yeast bioassay.

Several molecular mechanisms for adaptation have been described for the yeast mating signal transduction pathway (1). Alterations to one or more of these mechanisms should serve to enhance the sensitivity of a bioassay by altering desensitization pathways and, therefore, prolonging the signal initiated by agonist binding to receptor. The effects of an *sst2* mutation on the sensitivity of the yeast bioassay were described previously (Example 6). As an alternative to the genetic modification at *sst2*, overexpression of the yeast *STE50* gene was predicted to have similar effects (2), although by a different mechanism of action (2, 3). The *STE50* gene was isolated and placed under the control of a strong constitutive promoter in a high-copy-number plasmid resulting in significant overexpression of the gene. Yeast engineered to respond to the mammalian hormone somatostatin through an expressed SSTR2 somatostatin receptor were found to exhibit a more robust response to hormone if *STE50* was overexpressed.

MATERIALS AND METHODS

Construction of *STE50* expression plasmid. Growth of bacterial strains and plasmid manipulations were performed by standard methods (4). The protein coding sequences for *STE50* were amplified by polymerase chain reaction (PCR) using oligonucleotides selected by examination of the published sequence (2). The sense

oligonucleotide (5'- GTCGACAAATCAG ATG GAG GAC GGT AAA CAG G -3') contained the translation start codon (underlined) and a *Sal*I restriction site and the antisense oligonucleotide (5'- GAGCTCA TTA GAG TCT TCC ACC GGG GG -3') contained the translation stop codon (underlined) and a *Sac*I restriction site. These oligonucleotides were used as primers in a standard PCR to amplify *STE50* from *Saccharomyces cerevisiae* genomic DNA. The 1,100 basepair amplification product was cloned into the pCR2 vector (Invitrogen Corp., San Diego, CA) and confirmed by DNA sequencing. The *STE50* sequences were then isolated on a *Sal*I-*Sac*I restriction fragment and cloned into a pADH expression vector (5), placing the expression of *STE50* under the control of the strong constitutive *ADH1* promoter. This plasmid was designated pOZ162.

Yeast strain construction. Growth and transformation of yeast strains were performed as described by Rose *et al.* (6). The SSTR2 somatostatin receptor expression strain LY268 (*MAT***a** *ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2*, pJH2, pLP82) was described in prior examples and by Price *et al.* (7). Strain LY268 was transformed with either the *STE50* expression plasmid pOZ162 or the pADH vector. These strains are denoted CY560 or CY562, respectively.

Bioassay. Bioassay of SSTR2 somatostatin receptor expressed in yeast was described in prior examples and by Price *et al.* (7). Briefly, yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2%) and lacking uracil, tryptophan and leucine (SCD-ura-trp-leu), washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2%)-ura-trp-leu liquid medium. Molten (52° C) SC Galactose (2 %)-ura-trp-leu-his agar medium (30 ml, adjusted to pH 6.8 by addition of KOH prior to autoclaving) was inoculated with 0.06 ml of the overnight

culture to produce a final cell density of approximately 10^5 cells/ml and poured in square (9 x 9 cm) petri plates. Sterile filter discs were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of somatostatin-14 (Bachem Bioscience Inc., Philadelphia, PA) or α mating pheromone (Sigma, St. Louis, MO). Plates were incubated at 30° C for 3 days.

RESULTS

The effect of *STE50* overexpression on the sensitivity of the yeast bioassay was examined by comparing strains differing only in the level of *STE50* expression (Fig. 26). Bioassay plates were made containing either the *STE50* overexpression strain CY560 or the control strain CY562. The responses of these strains to somatostatin or yeast pheromone were examined as described in Materials and Methods. Both strains had very weak responses to yeast pheromone because the wild-type yeast Gα gene, *GPA1*, had been disrupted and functionally replaced by a gene expressing a chimeric Gpa1/$G\alpha_{i2}$ protein (7). This chimeric Gα protein does not efficiently interact with the yeast pheromone receptor. However, the response of these cells to somatostatin is strong (Fig. 26). As predicted, the overexpression of *STE50* resulted in a more robust response (Fig. 26a). These data demonstrate that the overexpression of *STE50* produces a hypersensitivity to ligands acting through G protein-coupled receptors coupled to the yeast signal transduction pathway, even if the ligand and receptor originate from a heterologous source.

REFERENCES CITED IN THIS EXAMPLE

1. **Sprague GF, Thorner JW.** Pheromone response and signal transduction during the mating process of

Saccharomyces cerevisiae. In: The molecular and cellular biology of the yeast *Saccharomyces*. EW Jones JR Pringle and JR Broach, eds. Cold Spring Harbor Laboratory Press, 1992.

2. **Ramezani-Rad M, Xu G, Hollenberg CP 1992** ***STE50***, a novel gene required for activation of conjugation at an early step in mating in *Saccharomyces cerevisiae*. Mol Gen Genet 236:145-154

3. **Chan RK, Otte CA 1982** Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by α factor and α factor pheromones. Mol Cell Biol 2:11-20

4. **Maniatus T, Fritsch EF, Sambrook J.** Molecular cloning. Cold Spring Harbor Laboratory Press, 1982

5. **Martin GA, Viskochil D, Bollag G, et al. 1990** The GAP-related domain of the neurofibromatosis type 1 gene product interacts with *ras* p21. Cell 63:843-849

6. **Rose MD, Winston F, Hieter P.** Methods in yeast genetics. Cold Spring Harbor Laboratory Press, 1990

7. **Price LA, Kajkowski EM, Hadcock JR, Ozenberger BA, Pausch MH 1995** Yeast cell growth in response to agonist-dependent activation of a mammalian somatostatin receptor. Submitted.

EXAMPLE 14

IDENTIFICATION OF COMPOUNDS WITH SOMATOSTATIN RECEPTOR AGONIST AND/OR ANTAGONIST PROPERTIES

Novel subtype-selective compounds with somatostatin agonist properties have significant therapeutic potential in the detection and treatment of various types of cancer. Compounds with somatostatin antagonist properties may be useful in promoting growth hormone release in agricultural species. Increased growth hormone release may lead to useful improvements in growth performance and carcass quality. To these ends, a yeast-based mechanism-based screening assay was developed to assay compounds for those that possessed desirable somatostatin agonist and/or antagonist properties.

Bioassay. A bioasssay designed to detect compounds with somatostatin agonist and/or antagonist properties was mobilized using a yeast strain (LY364 MAT**a** ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpalΔhisG far1ΔLYS2 FUS1-HIS33 sst2ΔADE2, pJH2, pLP82) that functionally expressed the rate SSTR2. The assay was accomplished using a modicication of a standard procedure. Y364 was grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and typtophan (SCD-ura-trp) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (55°C) SC Galactose (2 %)-ura-trp-his agar medium (150 ml, adjusted to pH 6.8 by addition of concentrated (2 x 10^4 cells/ml) and plated in square (500 cm^2) petri plates For assay of antagonists, somatostatin (20 nM S-14) was added to the molten agar prior to pouring. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10µl of sterile water containing candidate compounds. Plates were incubated at 30° C for 3 days.

Results. Active compounds from a primary screen were reassayed and the results displayed in Figure 27. The

left hand panel displays the results of an assay for compounds with somatostatin agonist properties. Four compounds exhibited substantial growth promoting activity expected of compounds with somatostatin agonist properties. The compounds found in the bottom left four positions are varying amounts of somatostatin applied as controls. The right hand panel displays the results of an assay for compounds with somatostatin antagonist activity. In the antagonist bioassay, somatostatin is added to the molten agar prior to pouring. In this way, all cells within the plate are induced to grow in response to somatostatin. As applied active compounds with antagonist properties diffuse into the agar medium and come into contact with the cells within, the growth response induced by somatostatin is interrupted, yielding a clear zone of inhibited growth. Several compounds exhibited detectable growth inhibiting properties.

EXAMPLE 15

Fusion of *STE2* sequences to the amino terminal of SSTR2 reduces signaling efficiency in response to somatostatin.

High level functional expression in yeast of G protein-coupled receptors in general, and the SSTR2 in particular, was a necessary prerequisite to the development of a useful biassay. King *et al.* reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient receptor expression in yeast. To test this hypothesis and the effect of *STE2* sequences on expression of the somatostatin receptor in yeast, the rat SSTR2 cDNA was placed under the control of the *GAL1* promoter in plasmids pJH1 and pJH2. These constructs confer inducible overexpression of Gal4p, the transcriptional activating protein for galactose-

inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane franctions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). In SSTR2 expression plasmid pJH1, DNA sequences encoding the first 13 amino acids of SSTR2 were replaced with coding sequence for the first 23 amino acids of STE2 (Fig. 11). The rate SSTR2 sequences were introduced into pJH2 without modification of the protein coding sequences. Yeast strains containing these constructs (LY322: *MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2, pJH1, pLP82; LY268: MATa ura3-52 trp1Δ63 his3Δ200 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2, pJH2, pLP82)* bear a plasmid (pLp82) that confers expression of a chimeric Gα protein composed of the proposed amino-terminal βγ-interaction domain from Gpa1p and carboxy-terminal receptor interaction domain from rat $G_{\alpha i2}$ under the control of the GPA1 promoter. The magnitude of the response of these strains to applied somatostatin (S-14) was measured (Fig. 28). LY268 cells exhibited a robust growth response to applied S-14, demonstrating that rat SSTR2 does not require *STE2* sequences to be functionally expressed in yeast (Fig. 28A) The growth response of LY268 cells was substantially greater than that exhibited by LY322 cells (Fig. 28B). The sole difference between these strains is the presence of *STE2* sequences in pJH1 found in LY322. Thus, replacement of the amino terminum of SSTR2 with the equivalent segment of *STE2* greatly reduces the efficiency of signalling in response to applied somatostatin. In spite of the observations of King *et al.*, heterologous G protein-coupled receptors expressed in yeast do not require amino-terminal protein coding sequences from any yeast protein for functional expression.

What is claimed is:

1. A transformed yeast cell comprising a first nucleotide sequence which codes for a heterologous G protein coupled receptor and a second nucleotide sequence which codes for all or a portion of a G protein complex.

2. The transformed yeast cell of Claim 1, wherein said first nucleotide sequence is selected from the group consisting essentially of β2-adrenrgic receptor, α2-adrenergic receptor, 5HT-1A receptor, muscarinic acetylcholine receptor, growth hormone releasing factor receptor, cholecystokinin receptor, adenosine receptor, and somatostatin receptor.

3. The transformed yeast cell of Claim 1, wherein said second nucleotide sequence codes for a Gα protein selected from the group consisting essentially of yeast Gα, heterologous Gα, and chimeric Gα.

4. The transformed yeast cell of Claim 2, wherein said second nucleotide sequence codes for a Gα protein selected from the group consisting essentially of yeast Gα, heterologous Gα, and chimeric Gα.

5. The transformed yeast cell of any of Claims 1-4 further comprising a pheromone-responsive promoter and a reporter gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith.

6. The transformed yeast cell of any of Claims 1-4 further comprising a pheromone-responsive promoter and a reporter gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith, wherein said reporter gene is

selected from the group consisting essentially of *HIS3*, G418r, *URA3*, *CYH2*, *LYS2*, *CAN1* and *LacZ* genes.

7. The transformed yeast cell of any of Claims 1-4 further comprising a reporter gene construct *FUS1-HIS3*.

8. The yeast cell of any of Claims 1-4 further comprising at least one gene mutation selected from the group consisting essentially of *fus3*, *far1*, *sst2*, *sgv1*, *ste2*, *ste3*, *pik1*, *msg5*, *sig1*, *afr1*, and STE50.

9. The yeast cell of Claim 8 wherein said mutation is a deletion.

10. The yeast cell of Claim 8 wherein said mutation is an overexpression.

11. The yeast cell of Claim 10 wherein said gene is *STE50*.

12. The yeast cell of Claim 3 further comprising a mutation at a *FAR1* gene in conjunction with at least one mutation at a gene selected from the group consisting essentially of *SST2*, *STE50*, *SGV1*, *STE2*, *STE3*, *PIK1*, *MSG5*, *SIG1*, and *AFR1*.

13. The yeast cell of Claim 12 further comprising a reporter gene construct.

14. The yeast cell of Claim 13 wherein said reporter gene is *FUS1-HIS3*.

15. The yeast cell of Claim 3 further comprising a mutation in a *SCG1/GPA1* gene.

16. The yeast cell of Claims 3 or 13 wherein said heterologous Gα subunit comprises a subunit selected from the group consisting essentially of a Gs subunit, Gi subunit, Go subunit, Gz subunit, Gq, G11, and G16.

17. The yeast cell of Claims 3 or 13 wherein said Gα subunit is a chimeric subunit.

18. The yeast cell of Claims 3 or 13 wherein said Gα subunit is a chimeric subunit comprising the amino terminal domain of yeast *GPA1/SCG1* and the carboxy terminal domain of a heterologous $G\alpha_{i2}$.

19. The yeast cell of Claims 3 or 13 wherein said Gα subunit is $G\alpha_{i2}$ and a Gβγ complex is an endogenous yeast Gβγ complex.

20. The yeast cell of Claim 1 further comprising at least a portion of a nucleotide sequence encoding for a yeast G protein coupled receptor positioned upstream to said nucleotide sequence encoding for said heterologous G protein receptor and operatively associated therewith, to encode for a chimeric G protein coupled receptor.

21. The chimeric receptor of Claim 20 wherein said nucleotide sequence encoding for all or a portion of a yeast G protein coupled receptor comprises a yeast gene selected from the group consisting essentially of the group consisting of *STE2* and *STE3*.

22. The yeast cell of Claim 21 further comprising a nucleotide sequence encoding a chimeric G protein comprising yeast nucleotide sequences and sequences froma heterologous G protein.

23. The yeast cell of any of Claims 3, 14, and 20 further comprising a heterologous Gβγ complex.

24. A chimeric G protein coupled receptor encoded by the nucleotide sequence of Claim 20.

25. A nucleotide expression vector capable of expressing a heterologous G protein-coupled receptor into a cell membrane of a yeast cell.

26. A nucleotide expression vector of Claim 25 further capable of expressing Gα, β, and γ proteins into a yeast cell.

27. A method of assaying compounds to determine effects of ligand binding to a heterologous G protein coupled receptor expressed on the surface of yeast cells comprising the steps of:

a) providing a culture of the transformed yeast cell of Claim 1;

b) contacting a selected compound to said cells to allow binding of said compound to the heterologous G protein receptor expressed on the cell surface; and

c) determining the growth of said cells as an indication of the activation of the heterologous G protein coupled receptor upon binding of said compound.

28. The method of assaying compounds of Claim 27 to determine effects on cell growth comprising:

(a) providing cultures of the yeast cell of Claim 1 by plating the yeast cells in agar medium

comprising a cognate agonist for a heterologously expressed receptor;

(b) applying selected compounds to the agar plate; and

(c) identifying compounds that inhibit agonist dependent activation of the heterologous receptor.

29. The transformed yeast cell of any of Claims 1-4 further comprising a reporter system selected from the group consisting essentially of heterologous adenylylcyclase, heterologous G protein-coupled potassium channel, and heterologous PLC-β.

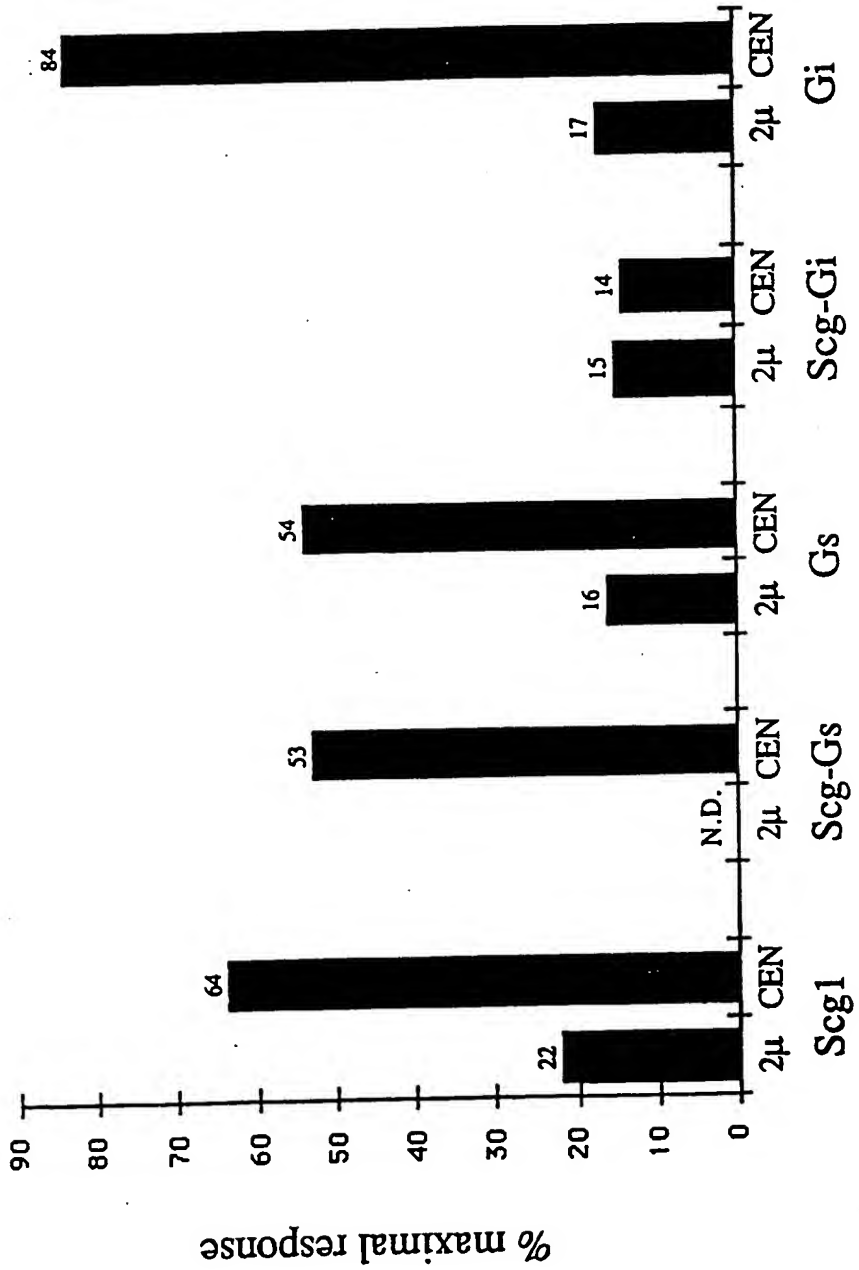

% maximal response
0
10
20
30
40
50
60
70
80
90
22
64
N.D.
53
16
54
15
14
17
84
2μ CEN
Scg1
2μ CEN
Scg-Gs
2μ CEN
Gs
2μ CEN
Scg-Gi
2μ CEN
Gi

FIG. 1

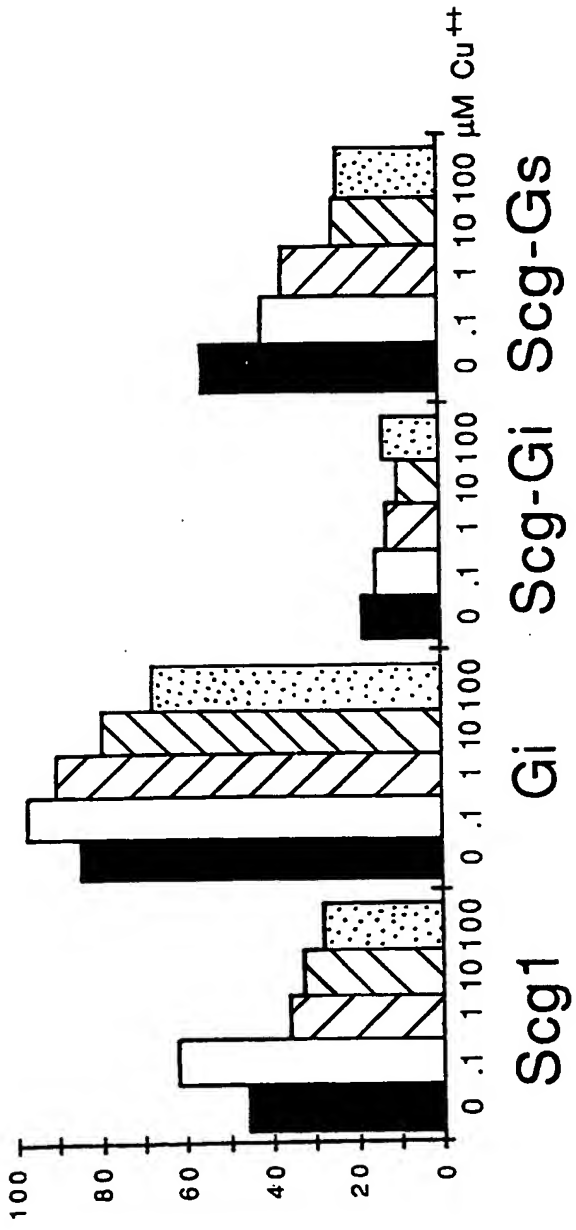

100
80
60
40
20
0
0 .1 1 10 100 0 .1 1 10 100 0 .1 1 10 100 0 .1 1 10 100 μM Cu++
Scg1
Gi
Scg-Gi
Scg-Gs

FIG. 2

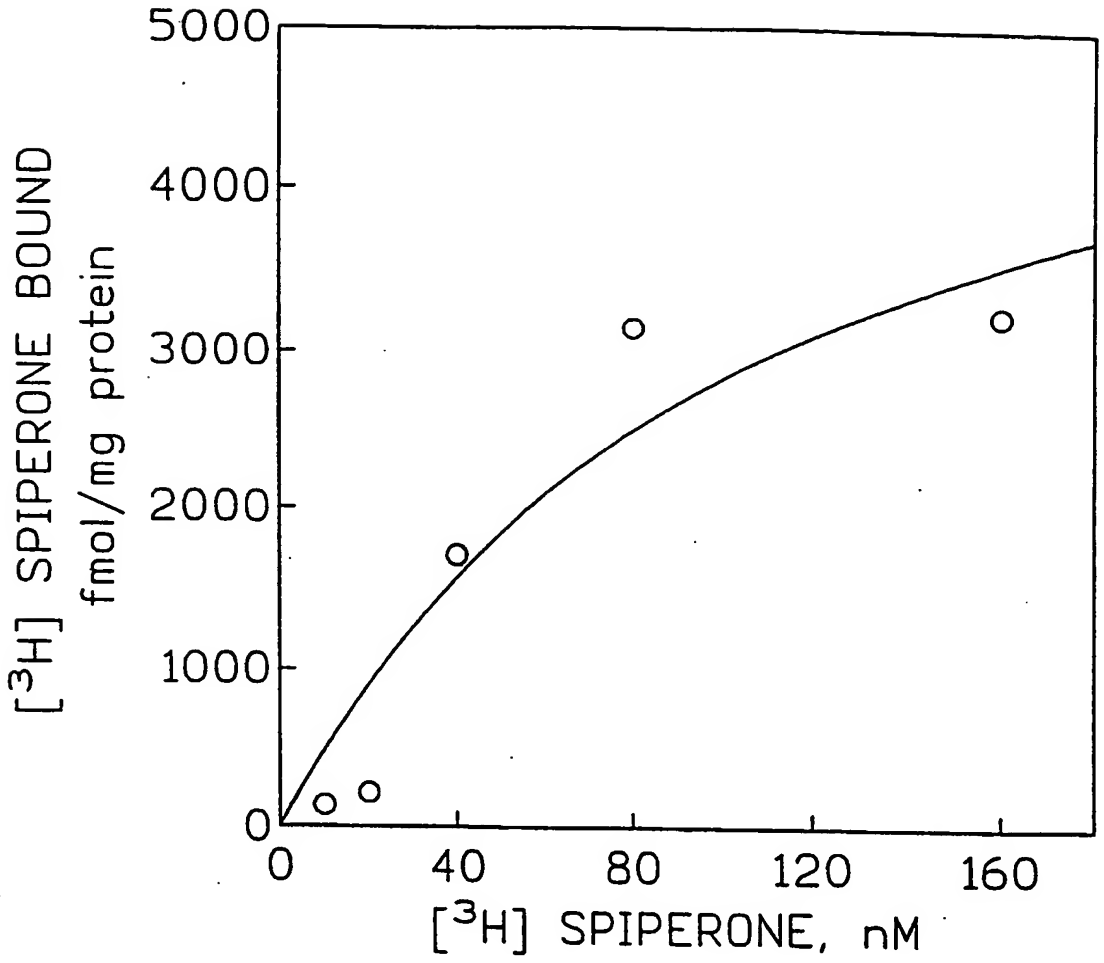

5000
4000
3000
2000
1000
0
0
40
80
120
160
[3H] SPIPERONE BOUND
fmol/mg protein
[3H] SPIPERONE, nM

FIG. 3

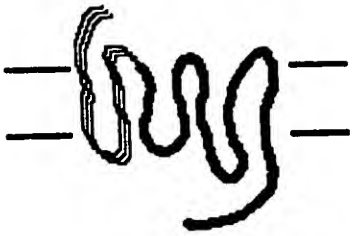
		Bmax (pmol/mg)
CHI11		3.1
CHI17		1.6
CHI18		0.7

FIG. 4

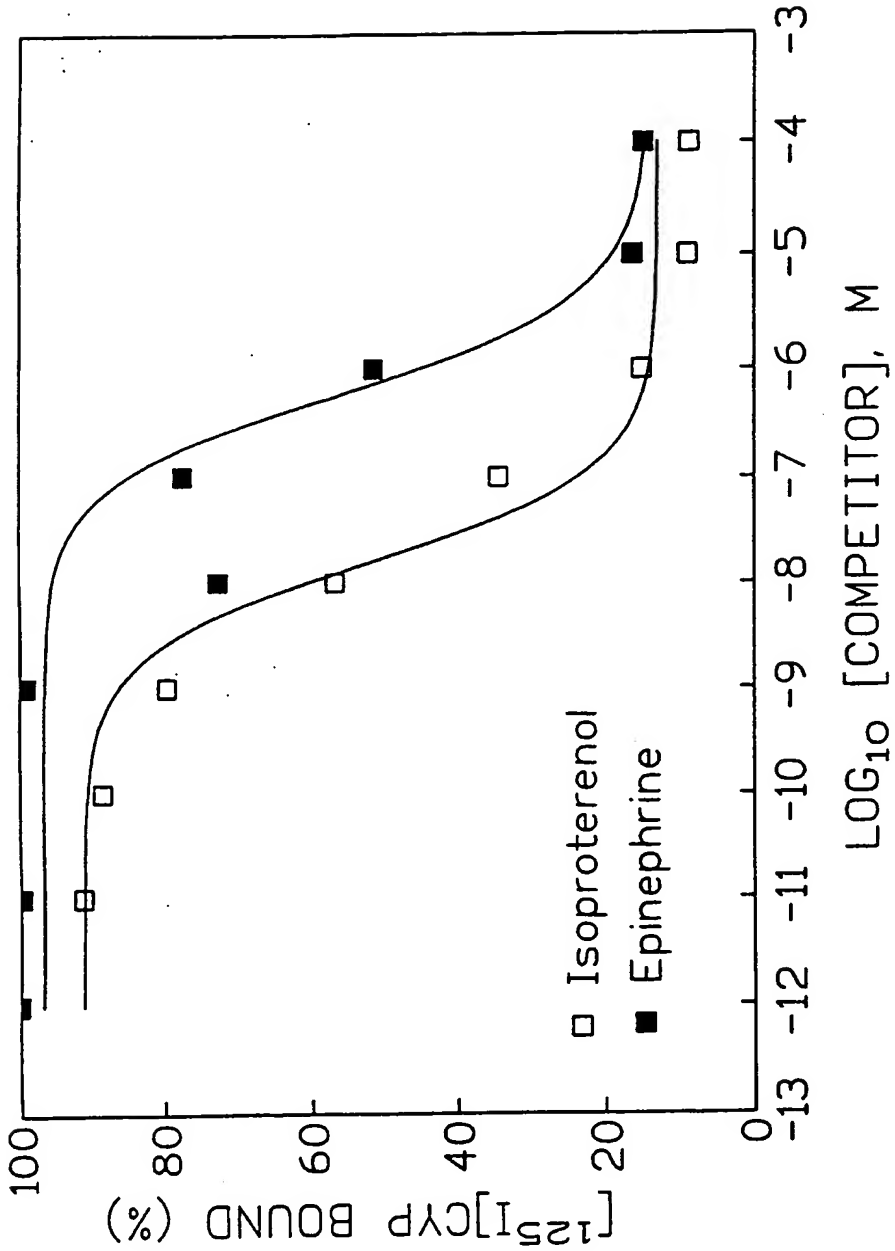

[125I]CYP BOUND (%)
100
80
60
40
20
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3
LOG10 [COMPETITOR], M
Isoproterenol
Epinephrine

FIG. 5

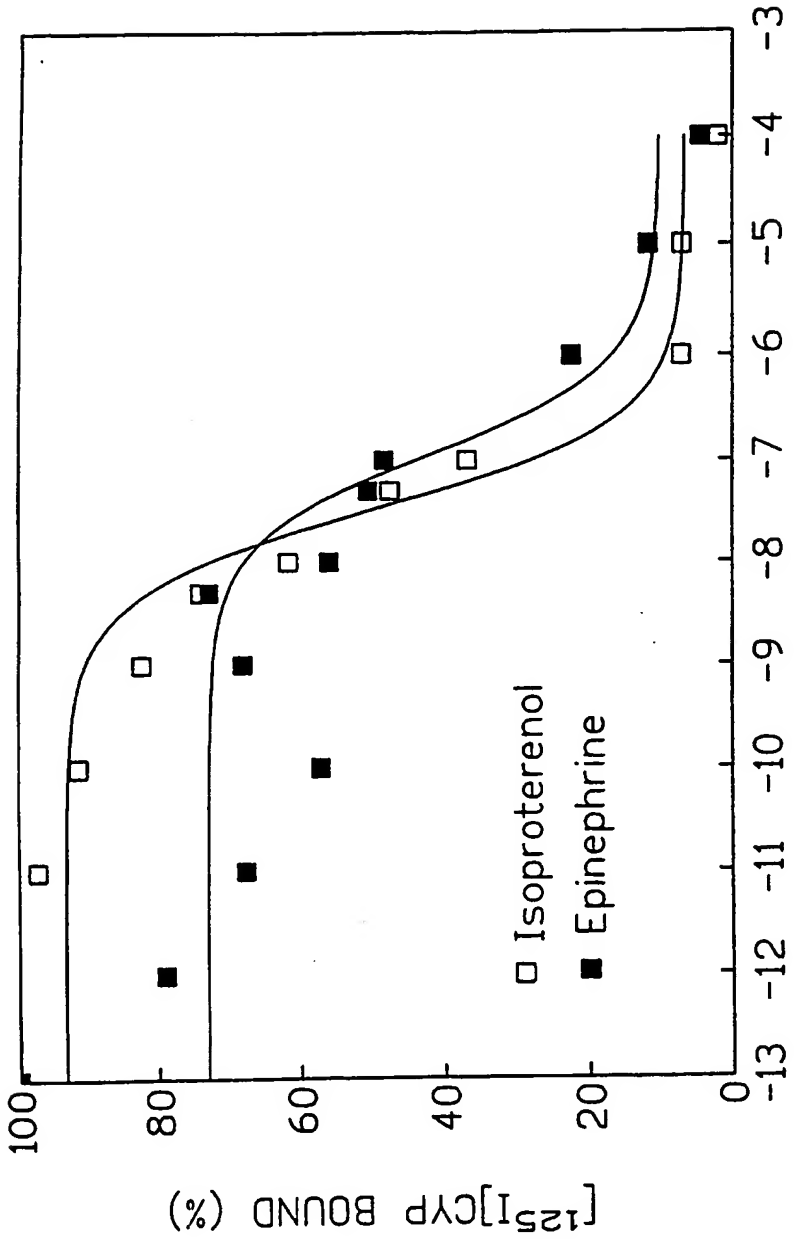

[125I]CYP BOUND (%)
100
80
60
40
20
0
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
LOG10 [COMPETITOR], M
Isoproterenol
Epinephrine

FIG. 6

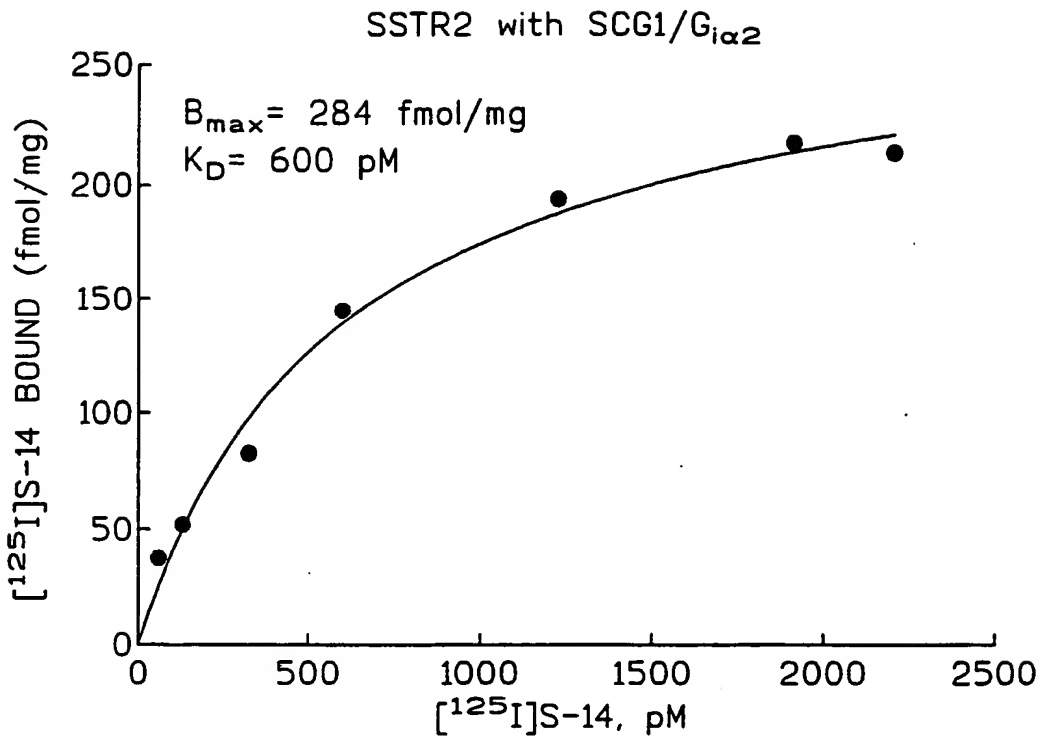

SSTR2 with SCG1/$G_{i\alpha 2}$
B_{max}= 284 fmol/mg
K_D= 600 pM
[^{125}I]S-14 BOUND (fmol/mg)
250
200
150
100
50
0
0
500
1000
1500
2000
2500
[^{125}I]S-14, pM

FIG. 7

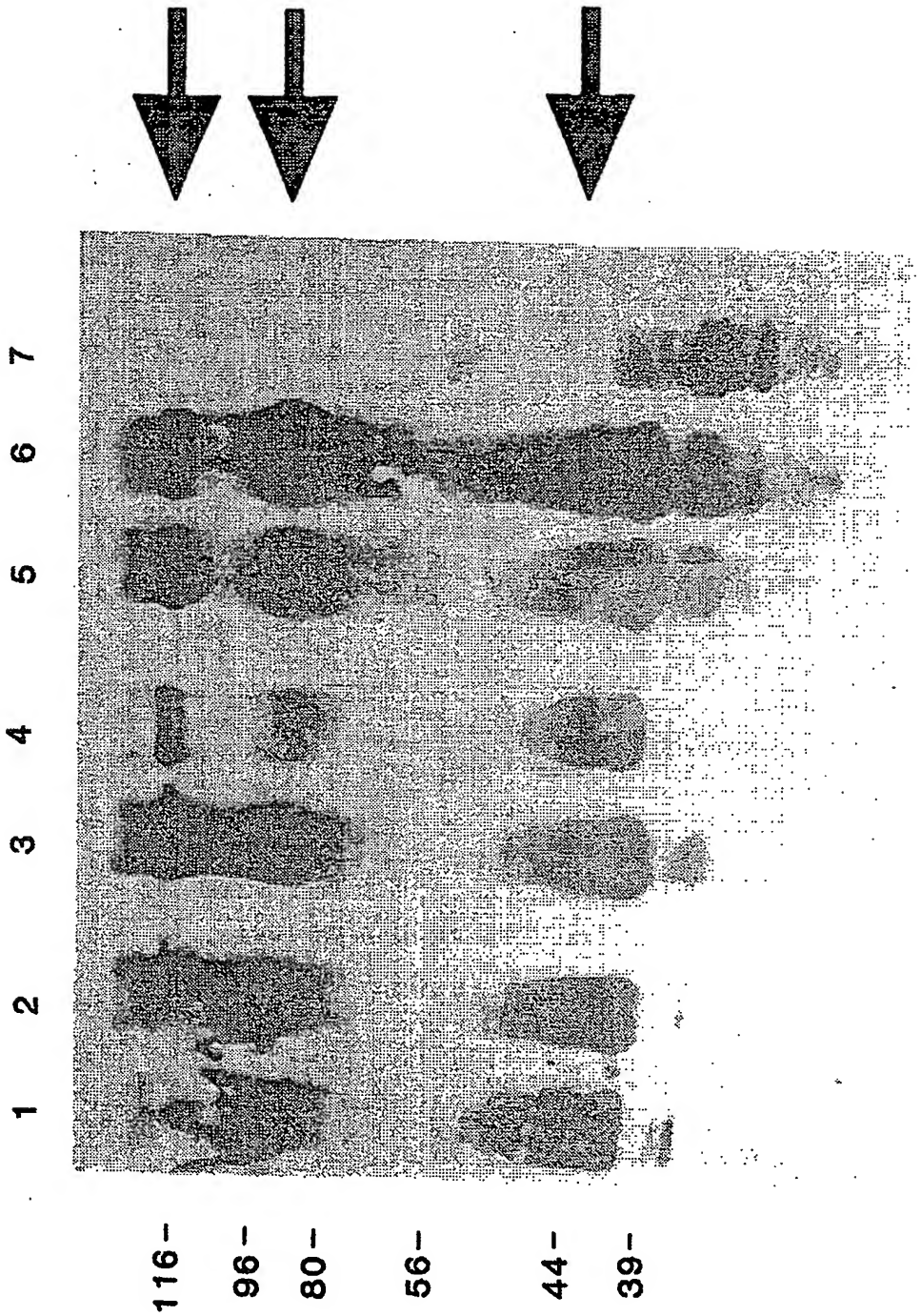


FIG. 8

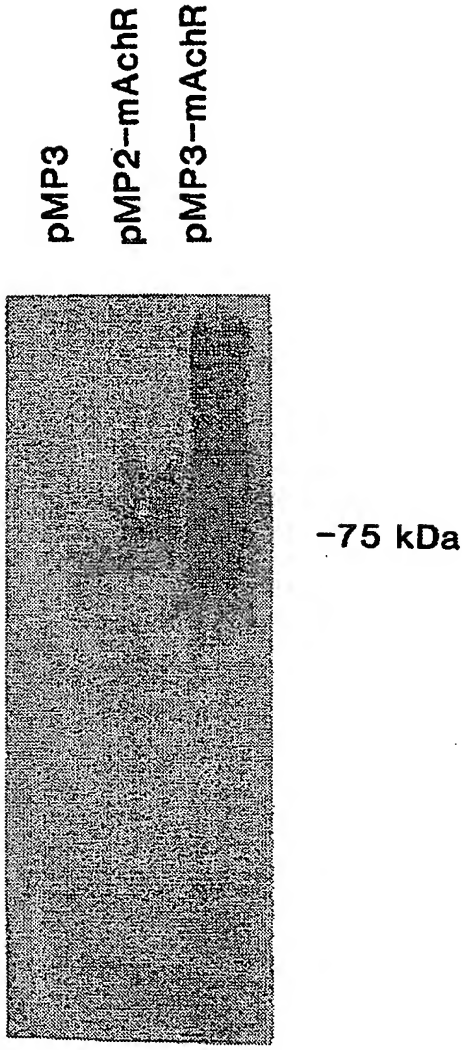


FIG. 9

FIG. 10

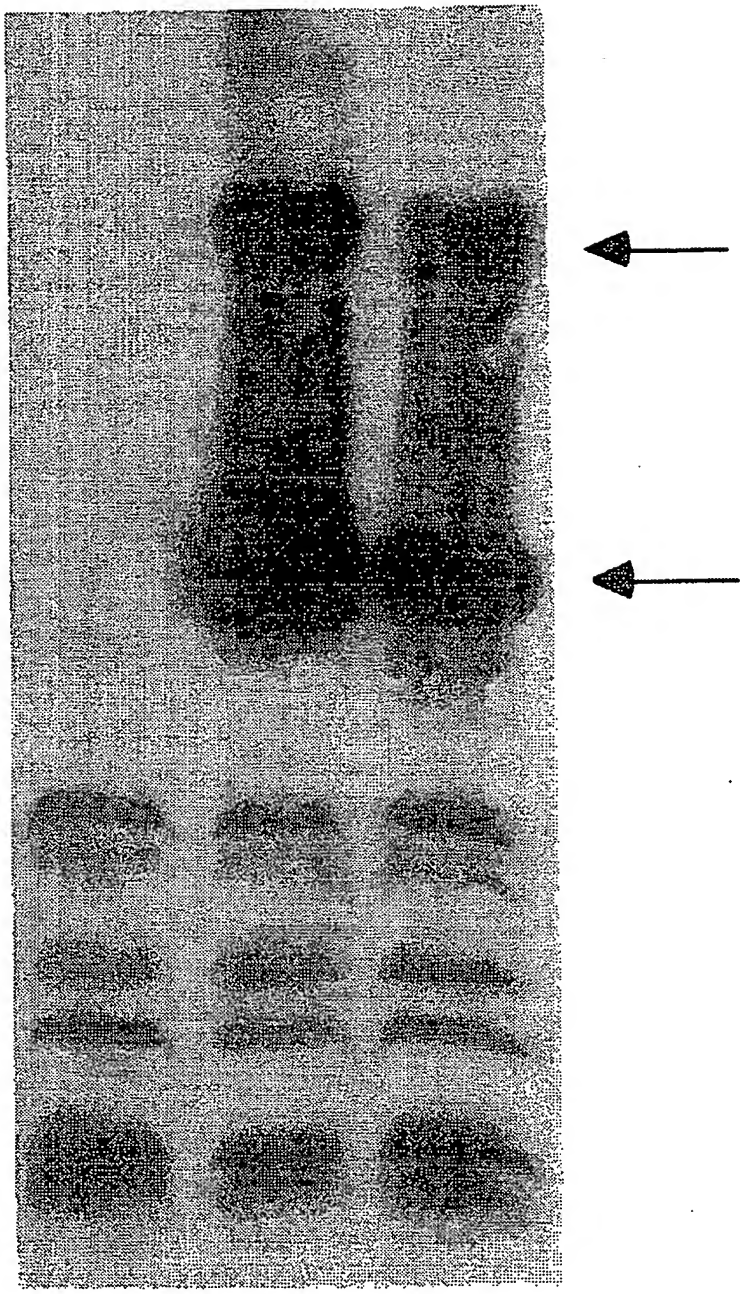
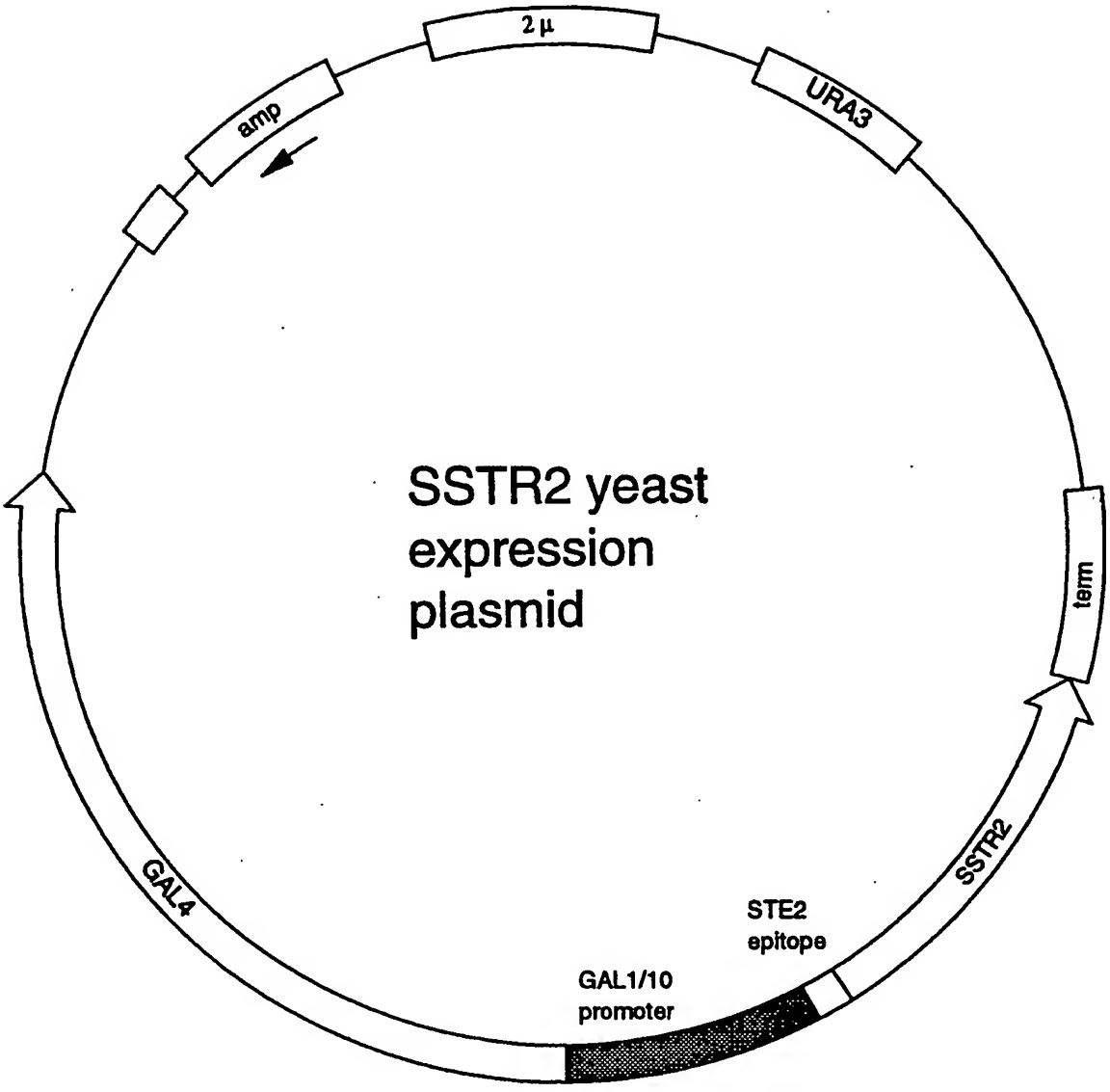


FIG. 11


2μ
URA3
amp
SSTR2 yeast
expression
plasmid
term
SSTR2
STE2
epitope
GAL1/10
promoter
GAL4

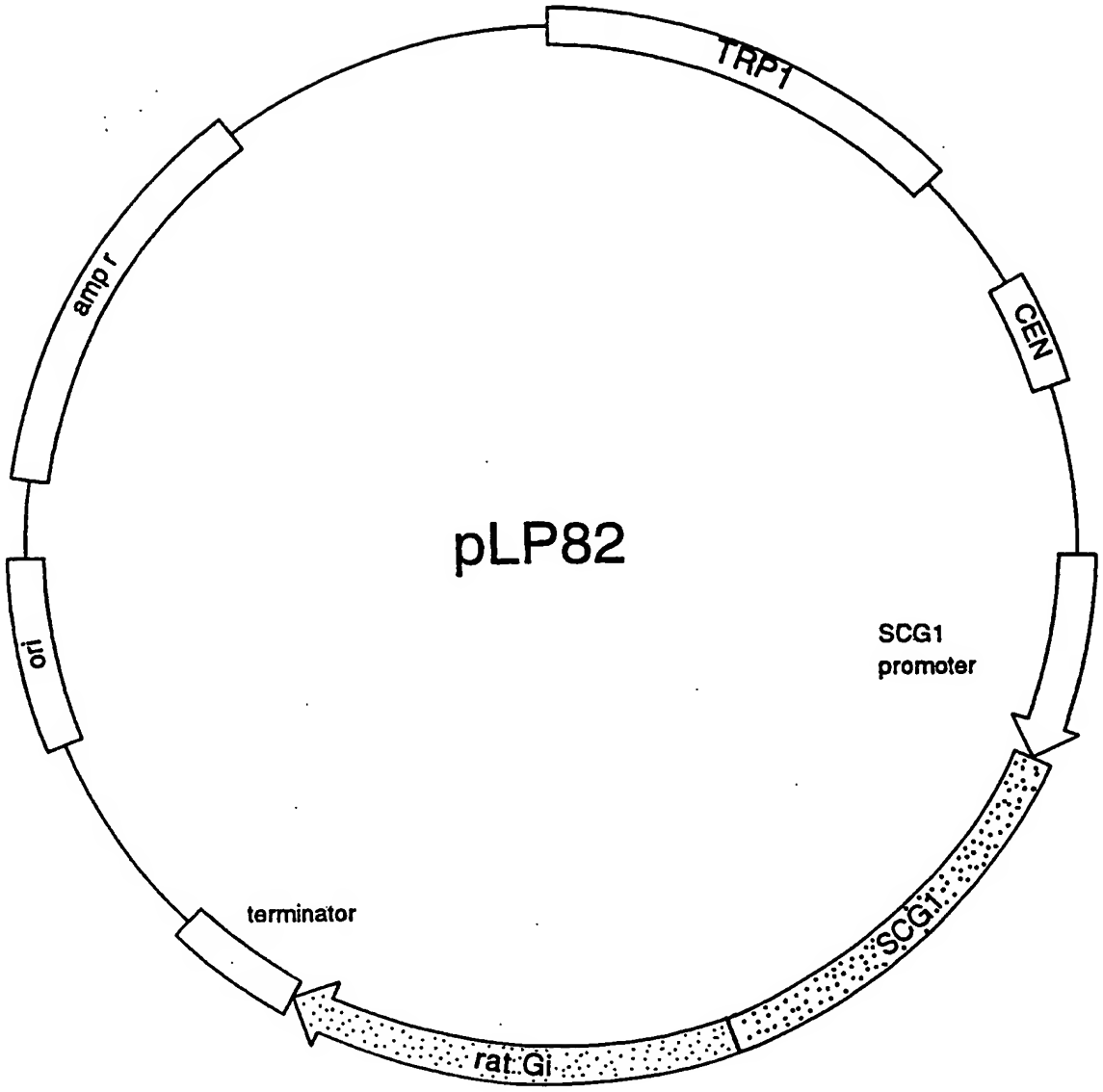

TRP1
CEN
SCG1
promoter
SCG1
rat Gi
terminator
ori
ampr
pLP82

FIG. 12

13/28

CEN pSCG1-Scg-$G\alpha_{i2}$

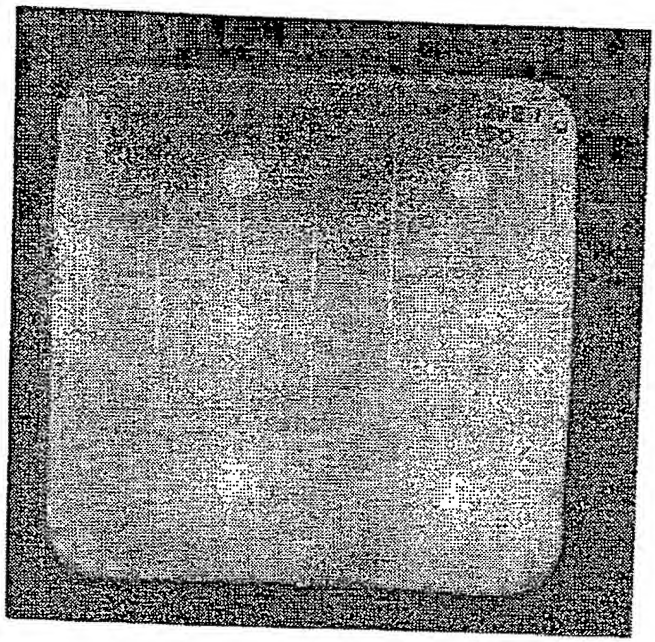


FIG. 13A

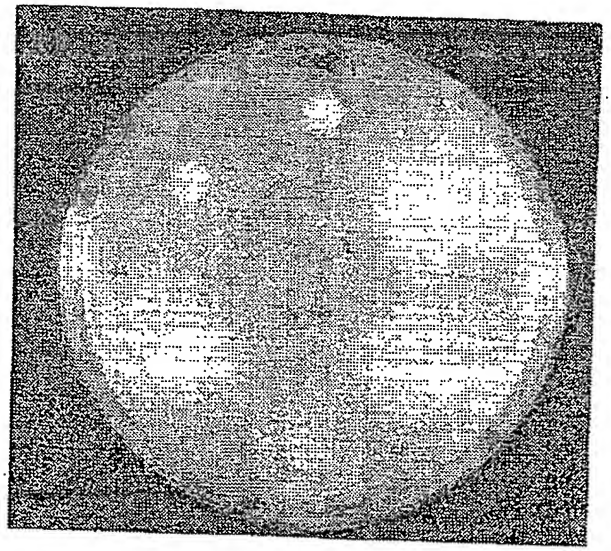


FIG. 13B

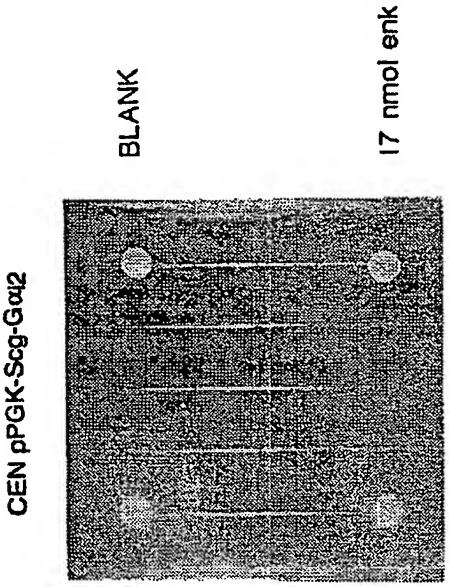


FIG. 14B

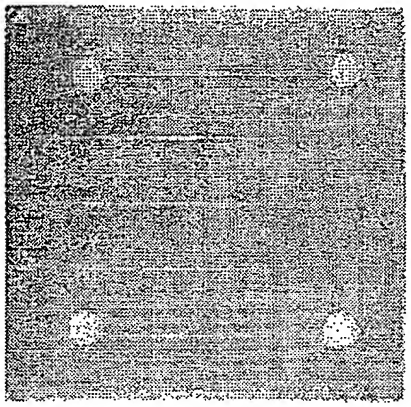


FIG. 14D

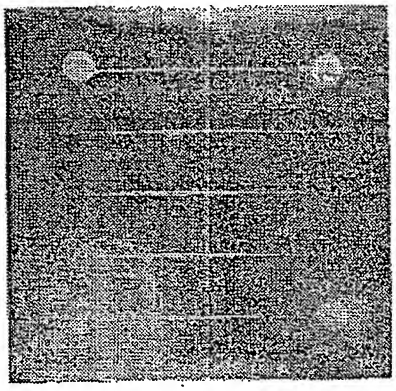


FIG. 14A

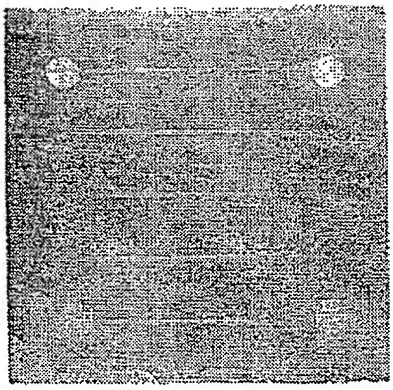


FIG. 14C

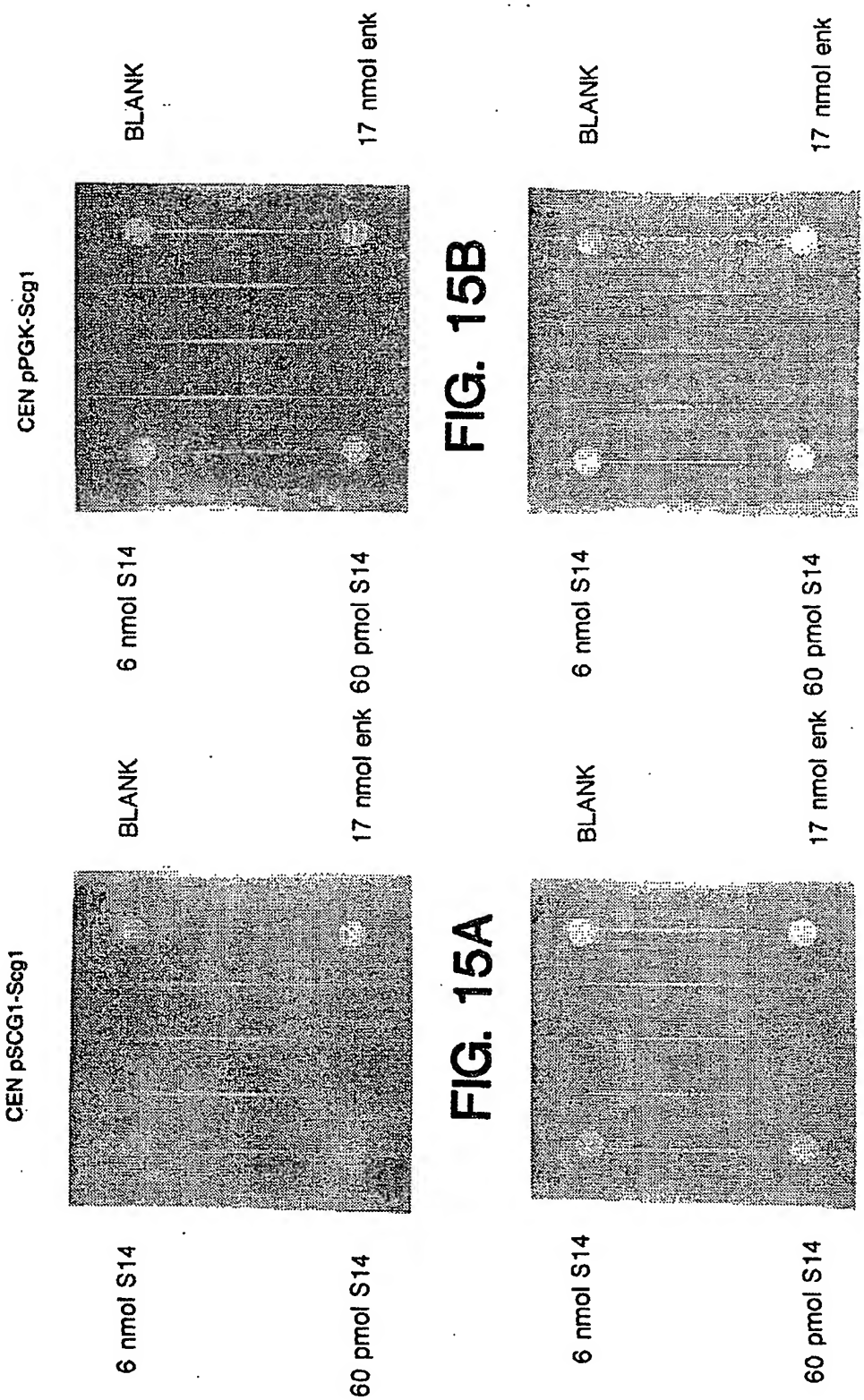


FIG. 15B

FIG. 15D

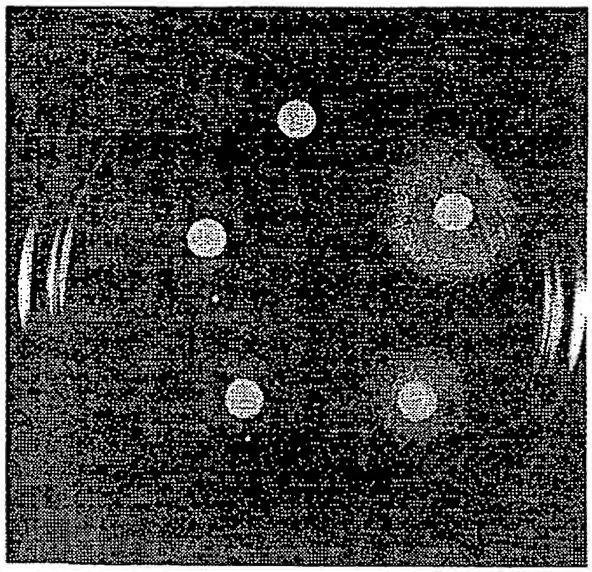
FIG. 15A

FIG. 15C

Carrier

0.001 nmol

1 nmol



0.01 nmol

0.1 nmol

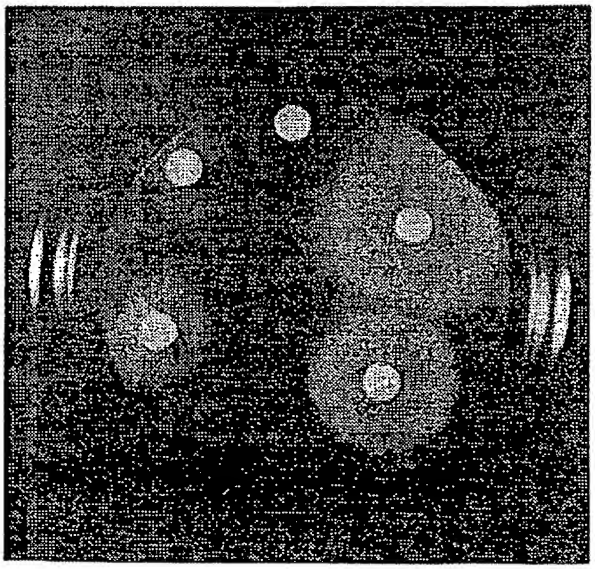
LY230 (SST2)

FIG. 16A

Carrier

0.001 nmol

1 nmol



0.01 nmol

0.1 nmol

LY238 (sst2)

FIG. 16B

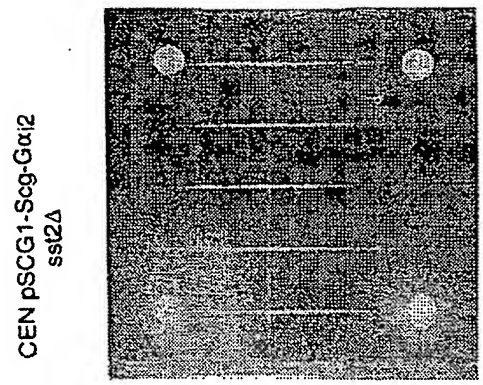


FIG. 17B

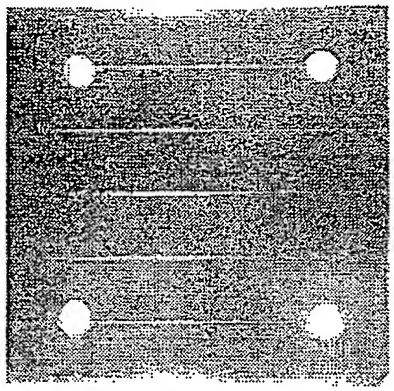


FIG. 17D

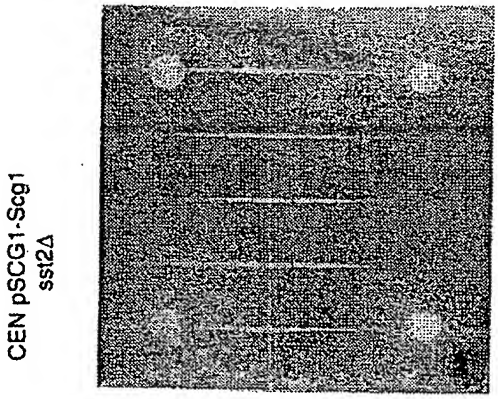


FIG. 17A

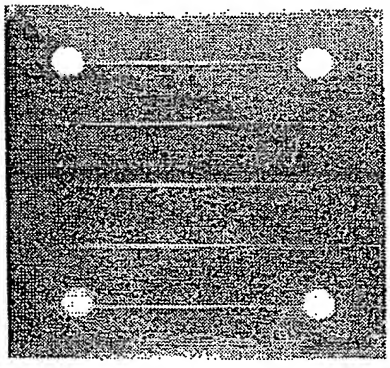


FIG. 17C

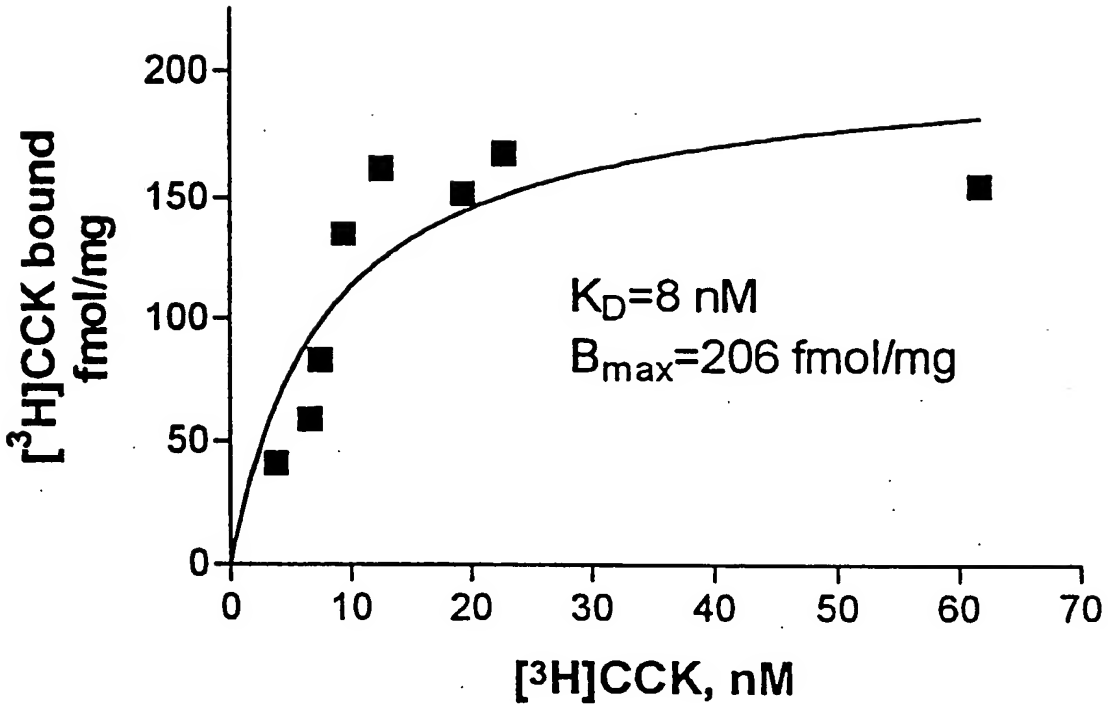

[3H]CCK bound fmol/mg
200
150
100
50
0
0
10
20
30
40
50
60
70
[3H]CCK, nM
KD=8 nM
Bmax=206 fmol/mg

FIG. 18

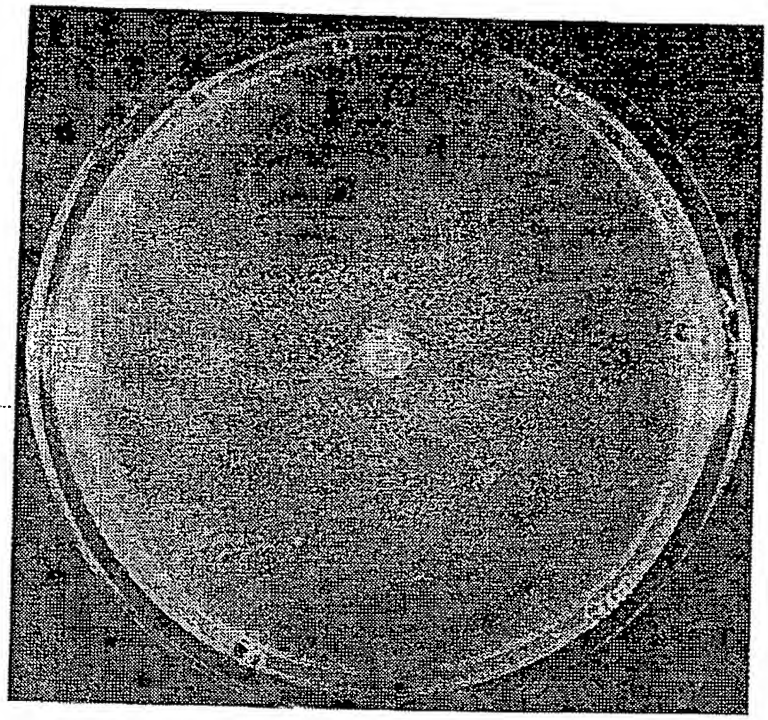


FIG. 19A

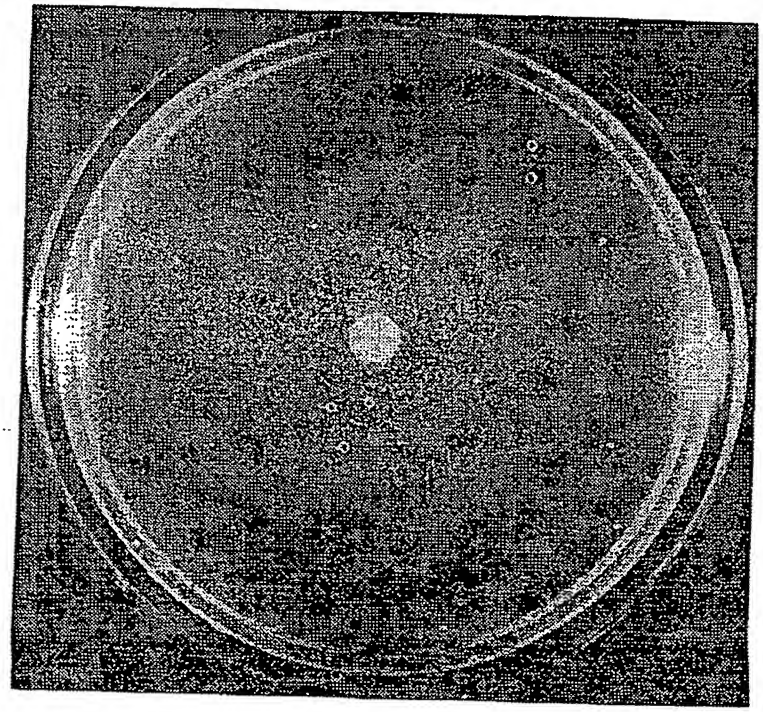


FIG. 19B

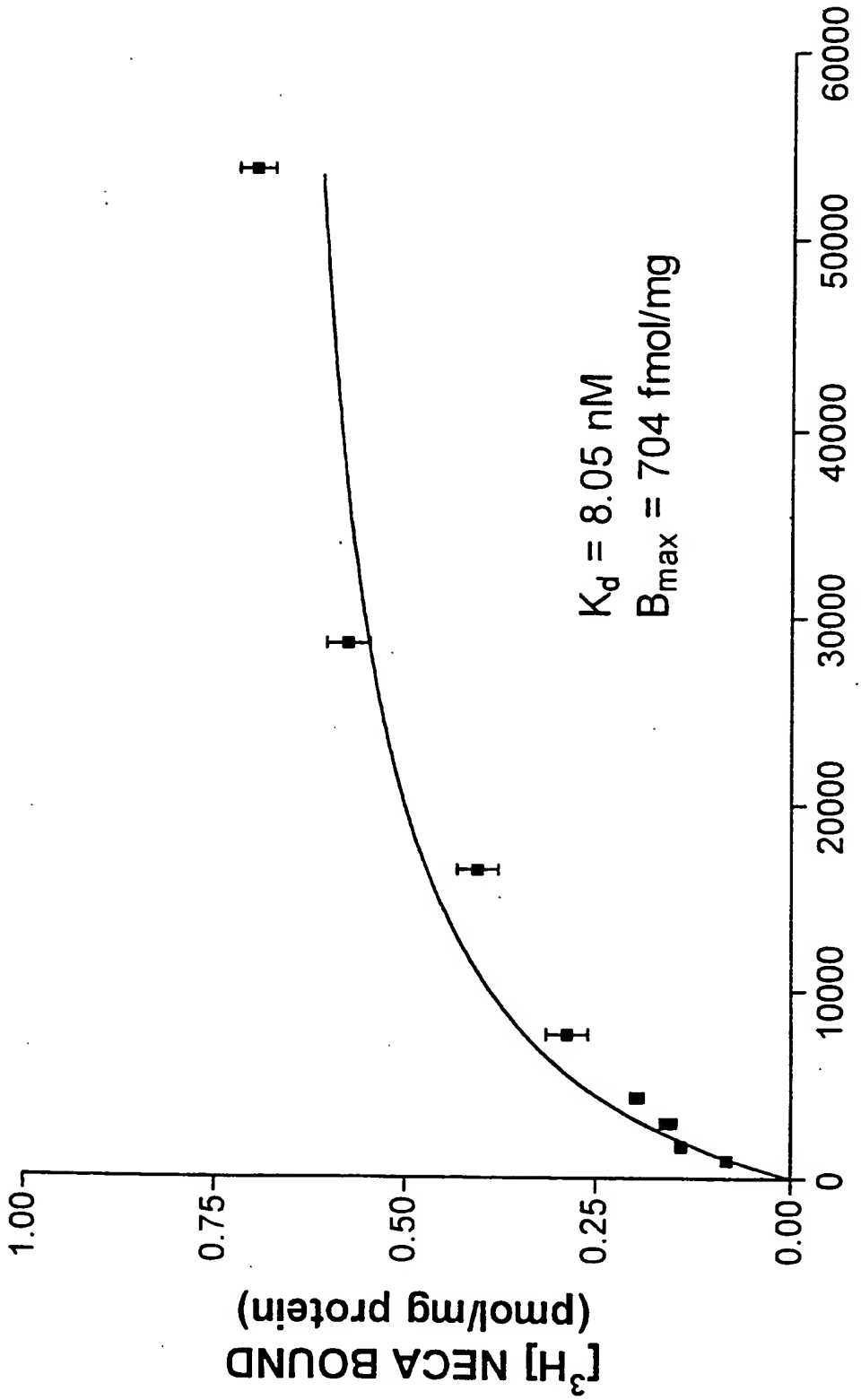

[3H] NECA BOUND
(pmol/mg protein)
1.00
0.75
0.50
0.25
0.00
0
10000
20000
30000
40000
50000
60000
[3H] NECA, pM
Kd = 8.05 nM
Bmax = 704 fmol/mg

FIG. 20

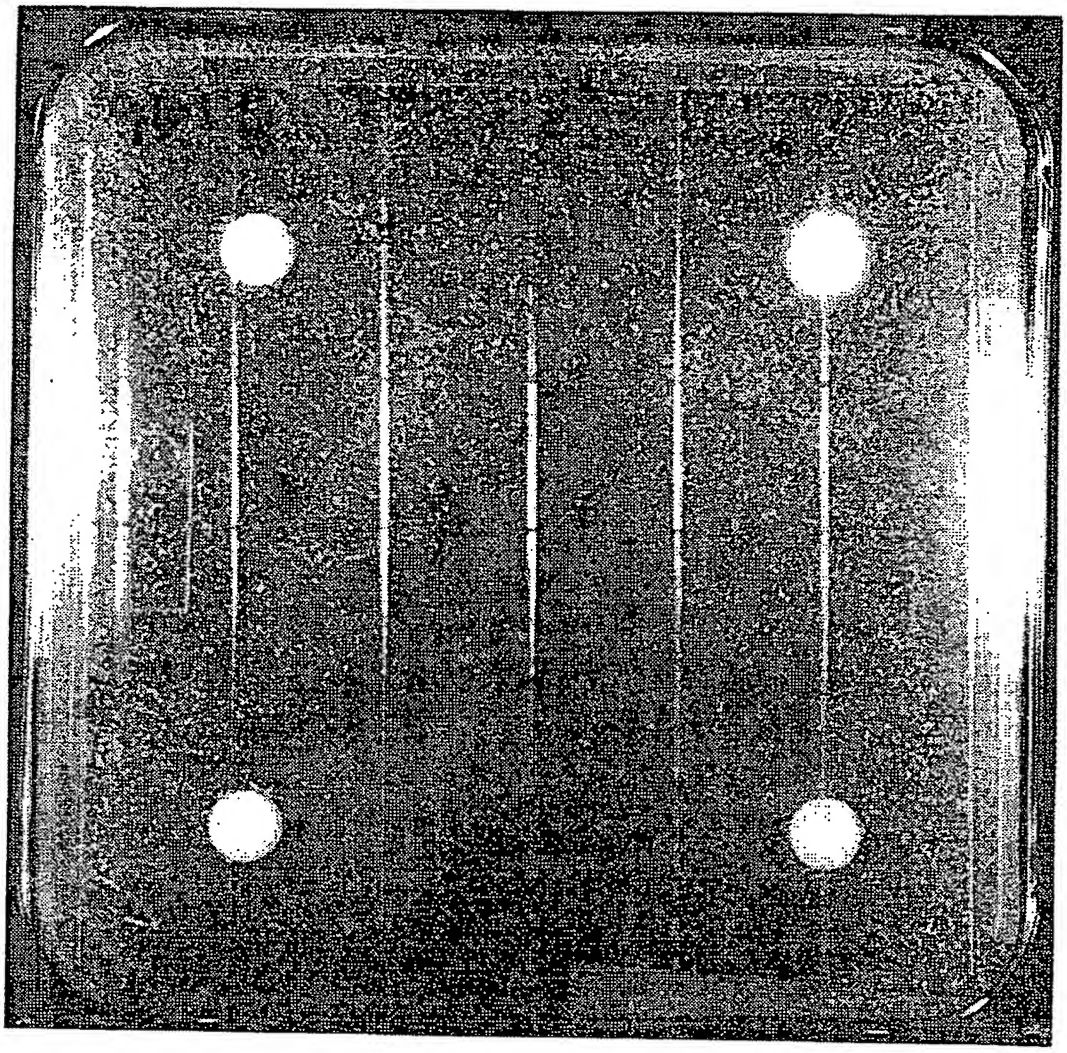


FIG. 21

A

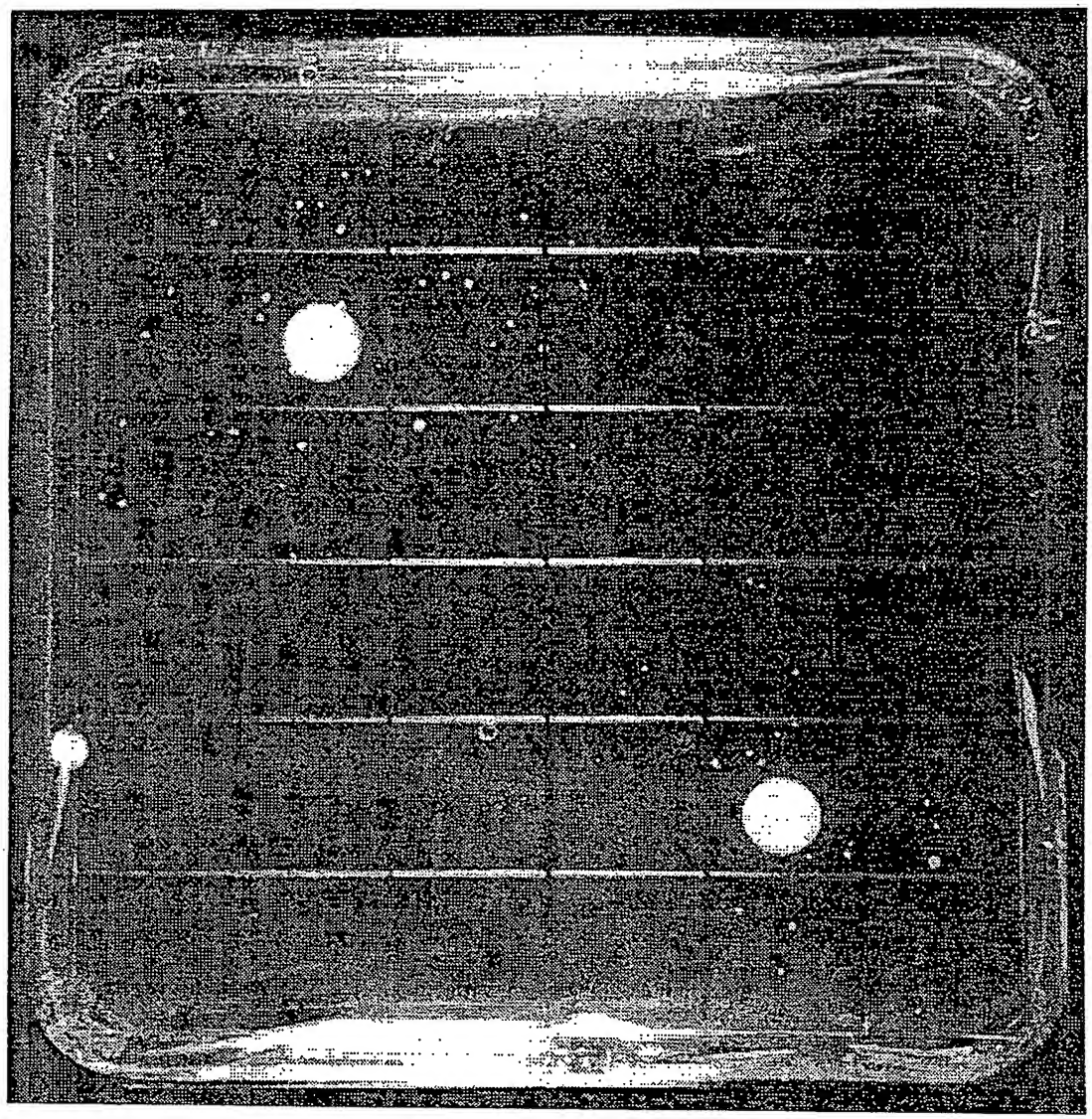


FIG. 22 B

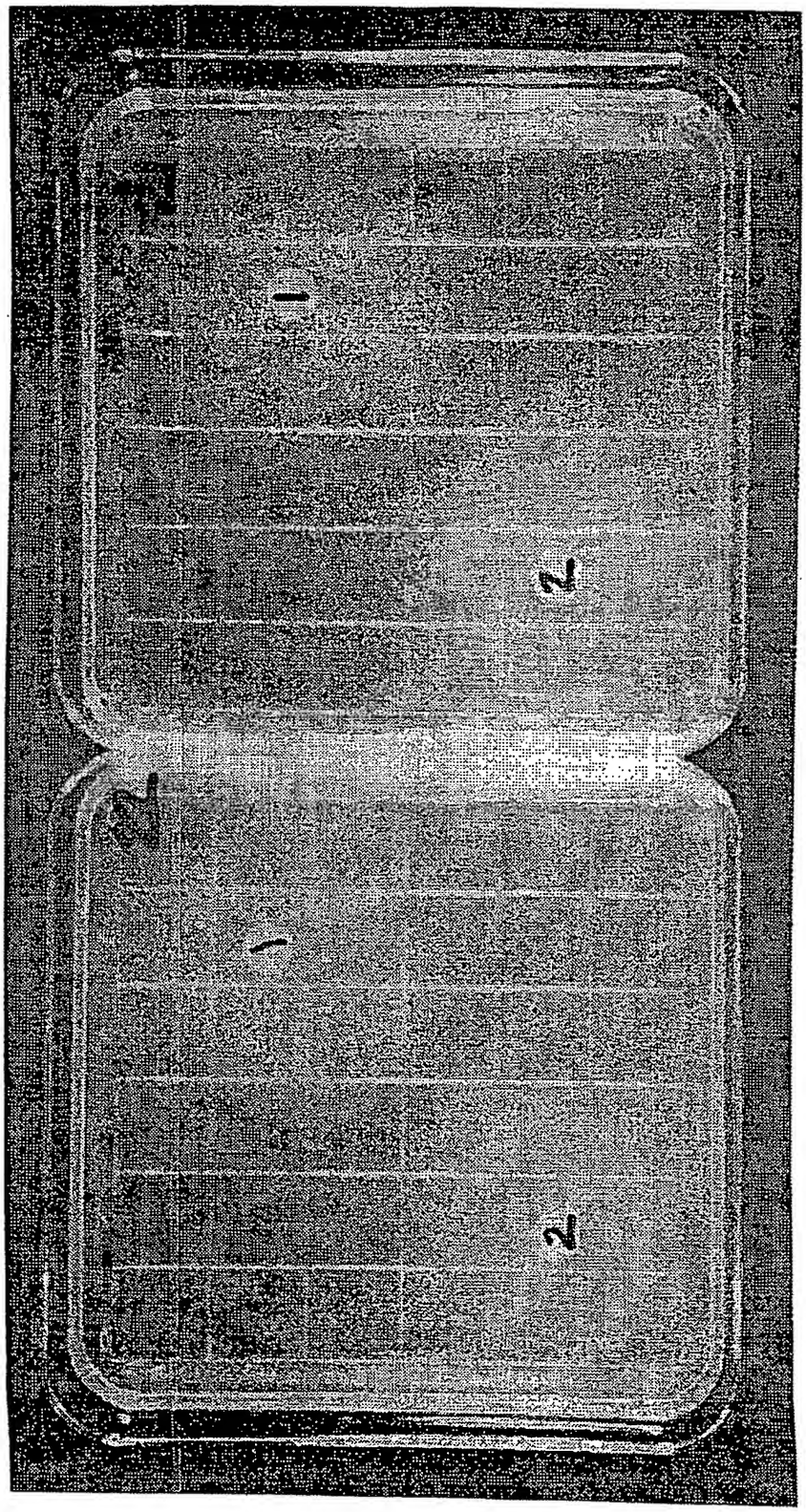


FIG. 23

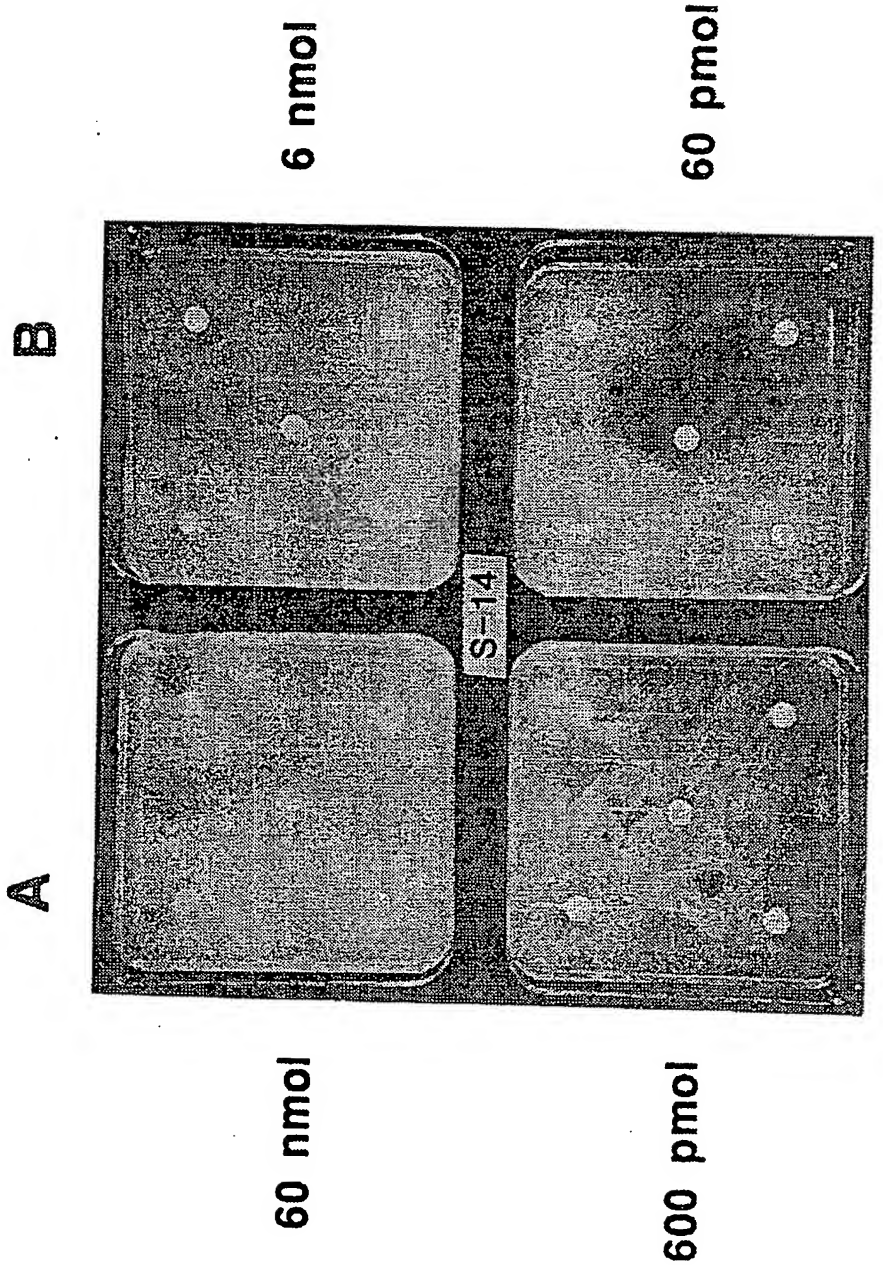


FIG. 24

FIG. 25B

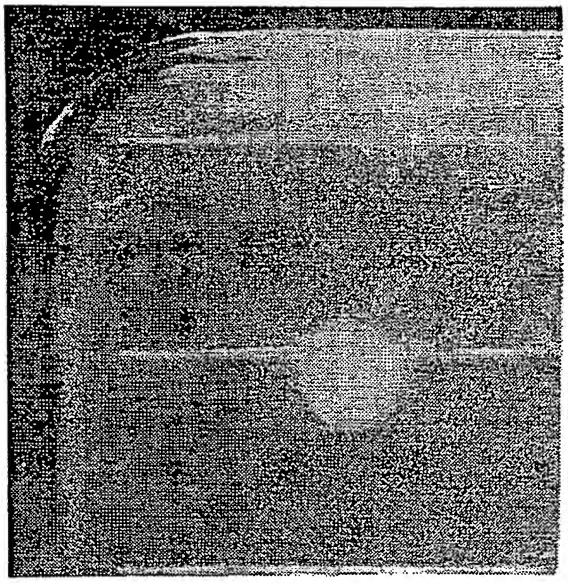


FIG. 25A

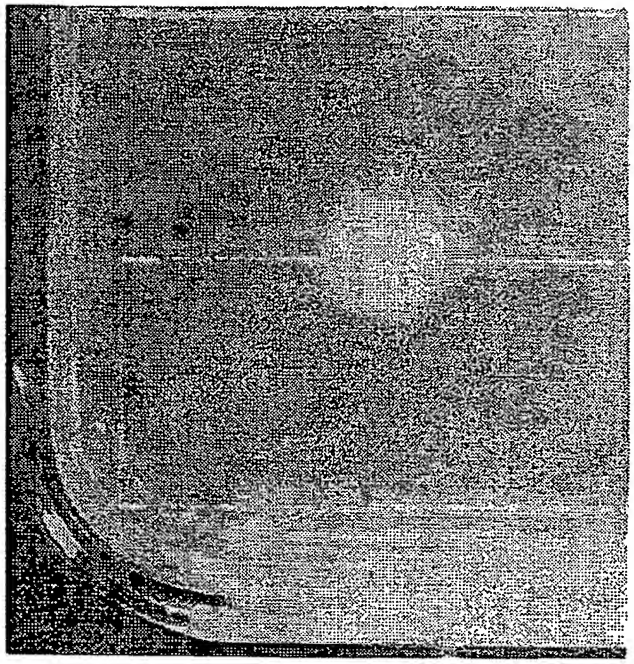


FIG. 26B

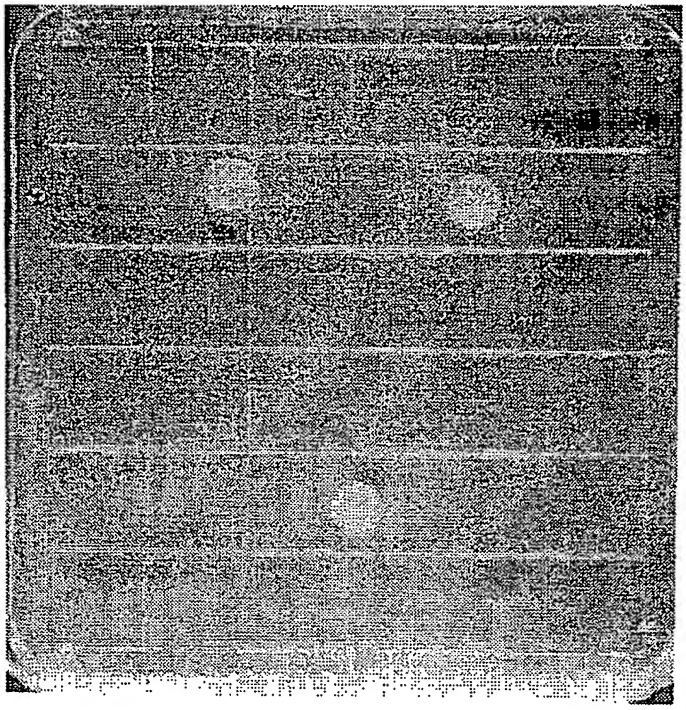


FIG. 26A

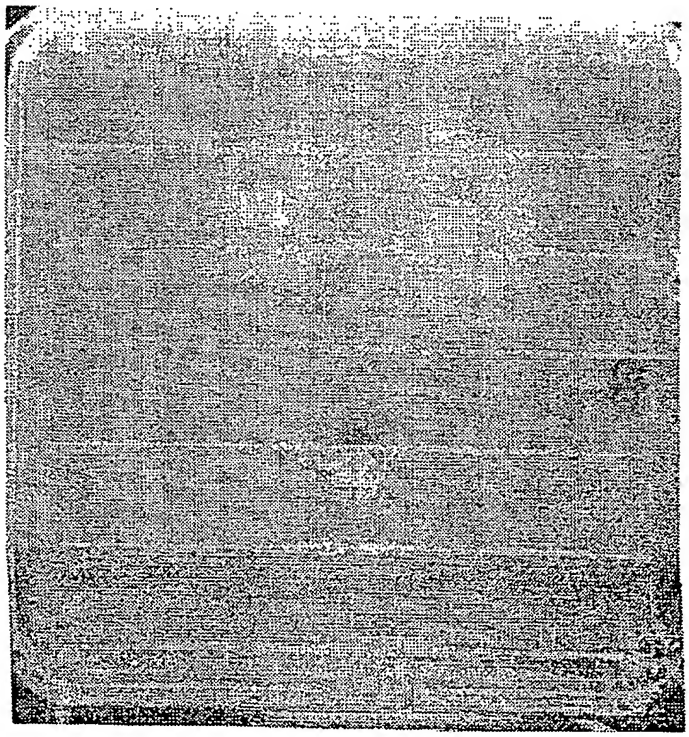
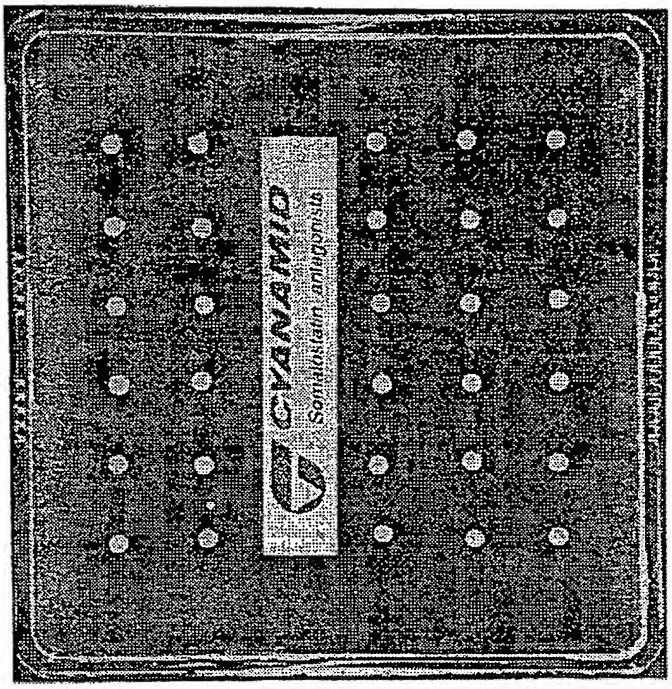
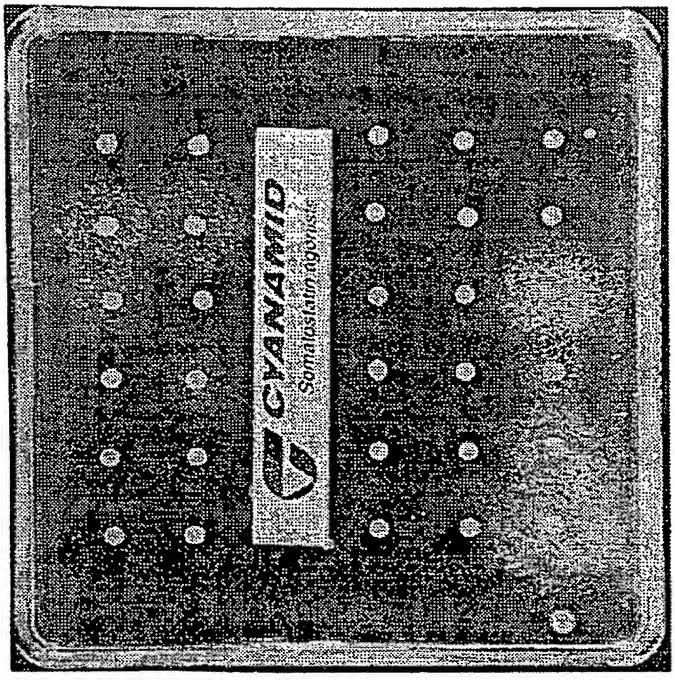


FIG. 27B



CL compounds (10 µl of 10 mg/ml solution) applied to each position.

FIG. 27A



CL compounds (10 µl of 10 mg/ml solution) applied to each position.

FIG. 28B

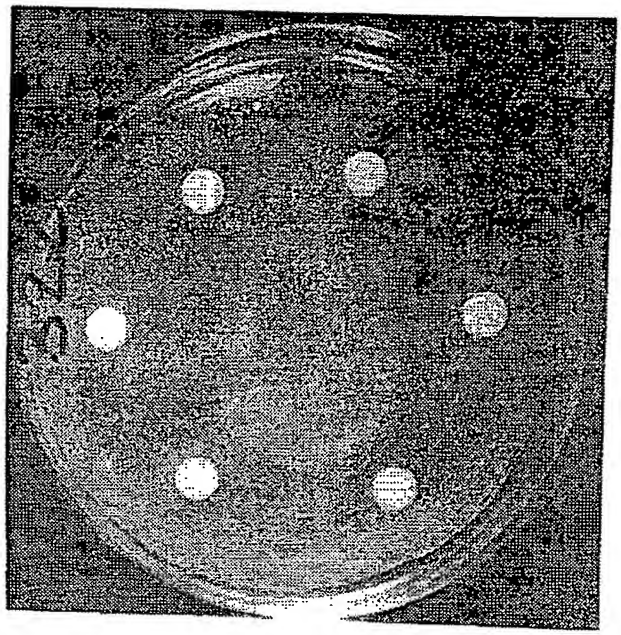
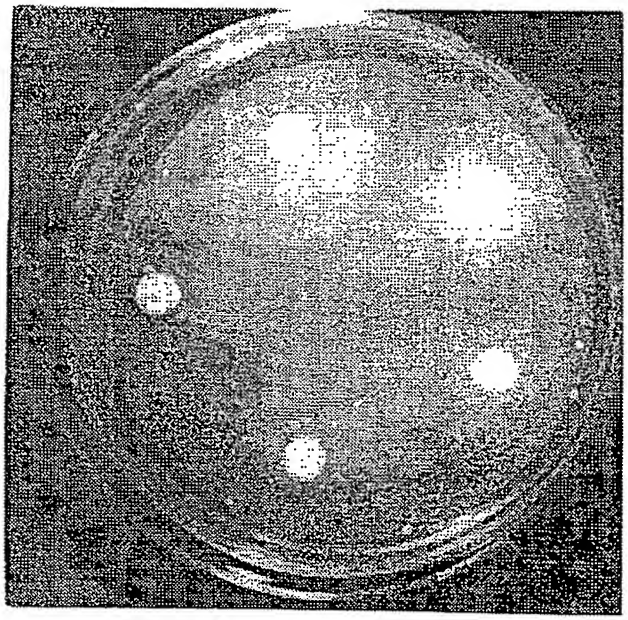


FIG. 28A



INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 95/02075

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C12N15/81 C12N1/19 C07K14/72 C12Q1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 05244 (DUKE UNIVERSITY) 2 April 1992 see page 2, line 16 - page 3, line 14 see page 4, line 35 - page 5, line 8 see page 5, line 29 - page 11, line 8; examples	1-6,15, 16, 20-22, 24-28
X	WO,A,91 12273 (ZYMOGENETICS, INC.) 22 August 1991 see page 3, line 32 - page 7, line 24	24,25

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 1995	04. 07. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/US 95/02075

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9205244	02-04-92	AU-B-	652576	01-09-94
		AU-A-	8511591	15-04-92
		CA-A-	2092717	14-03-92
		EP-A-	0548165	30-06-93
		JP-T-	6500693	27-01-94
WO-A-9112273	22-08-91	US-A-	5284746	08-02-94
		AU-A-	7333691	03-09-91
		EP-A-	0594594	04-05-94
		JP-T-	5504258	08-07-93